"ISOLATION, CHARACTERIZATION, ELUCIDATION OF ISOLATED PHYTO CONSTITUENT AND SCREENING OF ANTI MICROBIAL AND ANTI OXIDANT ACTIVITY OF *DELONIX REGIA* (BOJ.EX.HOOK) RAF LEAVES."

Dissertation submitted to

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Department of Pharmaceutical Chemistry
Madurai Medical College
Madurai – 625 020.

Prof.Mr.M.CHANDRAN, M.Pharm.,
Professor and Head of the Department,
Department of Pharmaceutical Chemistry,
Madurai Medical College,
Madurai.

CERTIFICATE

This is to certify that the Dissertation entitled **"Isolation, Characterization, elucidation of Isolated Phyto constituent and screening of anti microbial and anti oxidant activity of *Delonix regia* (BOJ.Ex.HOOK) RAF leaves."** by **Miss.N.ASTALAKSHMI** in the Department of Pharmaceutical Chemistry, Madurai Medical College, Madurai – 625 020, in partial fulfillment of the requirements for the Degree of Master of Pharmacy in Pharmaceutical Chemistry under my guidance and supervision during the academic year 2007-2008.

This dissertation is forwarded to The Controller of Examination, The Tamil Nadu Dr.MGR Medical University, Chennai.

Station: Madurai (**Prof.M.Chandran**)

Date:

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I hereby dedicate this little piece of work to Almighty.

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INTRODUCTION

Plants have been utilized as medicine for thousands of years. These medicines initially took the form of tinctures, teas, powders and other herbal formulations. The specific plants to be used and methods of application for particular ailments were passed down through oral history. In more recent history, the use of plants as medicine has involved the isolation of active compounds, beginning with the isolation of morphine from opium in the early 19th century. Drug discovery from medicinal plants led to the isolation of early drugs such as cocaine, codeine, digitoxin and quinine in addition to morphine of which some are still in use. Isolation and characterization of pharmacologically active compounds from medicinal plants continue today. More recently, drug discovery techniques have been applied to the standardization of herbal medicines to elucidate analytical marker compounds[1-3].

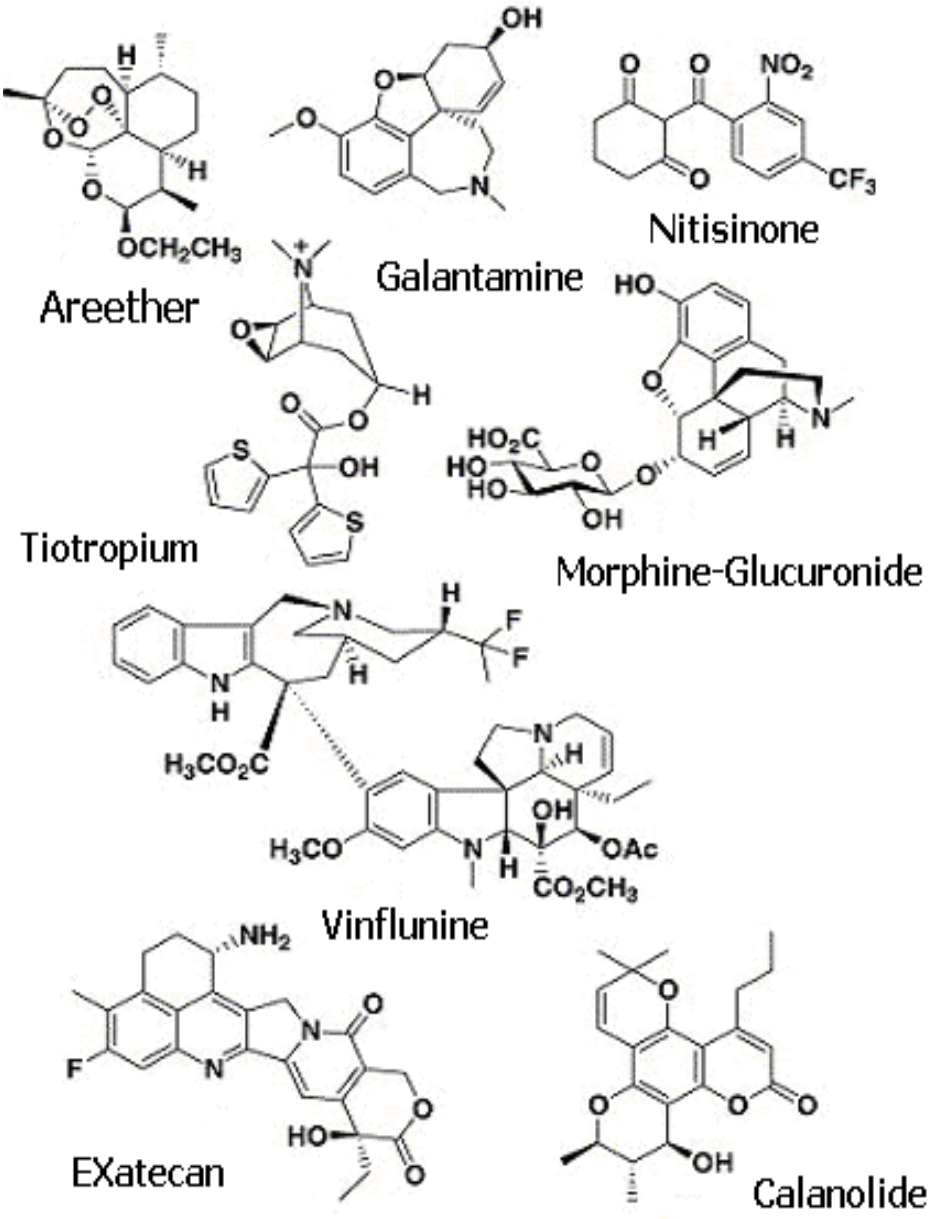
Importance of medicinal plants in drug discovery

Numerous methods have been utilized to acquire compounds for drug discovery including isolation from plants and other natural sources, synthetic chemistry an molecular modeling. Despite the recent interest in molecular modeling, combinatorial chemistry and other synthetic chemistry technique by pharmaceutical companies and funding organizations, natural products and particularly medicinal plants remain an important source of new drugs, new drug leads and new chemical entities (NCEs). In both 2001 and 2002, approximately one quarter of

the best selling drugs worldwide were natural products or derived from natural products[4-6].

There are also four new medicinal plant-derived drugs that have been recently introduced to the US market.

Fig. 01. EXAMPLES OF NEW MEDICINAL PLANT DRUGS RECENTLY INTRODUCED TO MARKET OR IN LATE-PHASE CLINICAL TRIALS.



Arteether (1, trade name Artemotil) is a potent anti-malarial drug and is derived from artemisinin, a sesquiterpene lactone isolated from *Artemisia annua L.* (Asteraceae), a plant used in traditional Chinese medicine (TCM). Other derivatives of artemisinin are in various stages of use or clinical trials as anti-malarial drugs in Europe [7]

Galantamine (2, also known as Galanthamine, trade name Remminyl) is a natural product discovered through an ethno botanical lead and first isolated from *Galanthus woronowii Losinsk* (Amaryllidaceae) in Russia in the early 1950s. Galantamine is approved for the treatment of Alzheimer's disease, slowing the process of neurological degeneration by inhibiting acetyl cholinesterase (AchE) as well as binding to modulating the nicotine acetylcholine receptor (nAchR)[8].

Nitisinone (3, trade name Orfadin) is a newly released medicinal plant-derived drug that works on the rare inherited disease, tyrosinaemia, demonstrating the usefulness of natural products as lead structures. Nitisinone is a modification of mesotrione; an herbicide based natural product leptpspermone, a constituent of *Callistemon citrinus Ctapf* (Myrtaceae). All three of these triketones inhibit the same enzyme, 4-hydroxy phenyl pyruvate and dehydrogenase (HPPD), in both human and maize. Inhibition of the HPPD enzyme in maize acts as an herbicide and results in reduction of plastoquinone and tocopherol biosynthesis, while in humans the HPPD enzyme inhibition prevents tyrosine catabolism and the accumulation of toxic bioproducts in the liver and kidneys[9].

Tiotropium (4, Trade name Spiriva) has recently been released to the United States market for the treatment of chronic obstructive pulmonary disease (COPD). Tiotropium is an inhaled anticholinergic branchodilator, based on ipratropium, a derivative of atropine that has been isolated from *Atropa belladonna Linn* (Solanaceae) and other members of the Solanaceae family. Tiotropium has shown increased efficacy and longer lasting effects when compared with other available COPD medications [(10)].

Compounds 5 – 7 (Fig. I) are all in Phase III clinical trials or registration and are subtle modifications of drugs currently in clinical use (Butler, 2004). M6G or Morphine — glucuronide (5) is a metabolite of morphine from *Papaver somniferum Linn* (Papaveraceae) and will be used as an

alternate pain medication with fewer side effects than morphine. Vinflunine (6) is a modification of vinblastine from *Catharanthus roseus Linn G. Don (*Apocynaceae) for use as an anticancer agent with improved efficacy [(11&12)].

Exatecan (7) is an analog of camptothecin from *Camptotheca accuminata Decne.* (Nyssaceae) and is being developed as an anticancer agent. Modifications of existing natural products exemplify the importance of drug discovery from medicinal plants as NCEs and as possible new drug leads[13].

Calanolide A (8) is a dipyranocoumarin natural product isolated from *Calophyllum lanigerun var. austrocoriaceum (Whitemore) PF Stevens* (Clusiaceae), a Malaysian raingorest tree. Calanolide A is an anti-HIV drug with unique and specific mechanism of action s a non-nuceoside reverse transcriptase inhibitor (NNRTI) of type-I HIV and is effective against AZT-resistant strains of HIV. Calanolide A is currently undergoing Phase II clinical trials [14].

Natural products have played an important role as new chemical entities (NCEs)- approximately 28% of NCEs between 1981 and 2002 were natural products or natural product-derived. Another 20% of NCEs during this time period were considered natural product mimics, meaning that the synthetic compound was derived from the study of natural products. Combining these categories, research on natural products accounts for approximately 48% of the NCEs reported from 1981 – 2002.

Natural products provide a starting point of new synthetic compounds, with diverse structures and often with multiple sterocentres that can be challenging synthetically. Many structural features common to natural products (e.g., chiral centers, aromatic rings, complex ring system, degree of molecule saturation and number and ratio of hetero atoms) have been shown to be highly relevant to drug discovery efforts [15].

Furthermore, since the escalation of interest in combinatorial chemistry and the subsequent realization that these compound libraries may not always be very diverse, many synthetic and medicinal chemists are exploring the creation of natural product and natural product like libraries that combine the structural features of natural products with the compound-generating potential of combinatorial chemistry. Drugs derived from medicinal plants can serve not only as new drugs themselves but also as drug leads suitable for optimization by medicinal and synthetic chemists[16-19].

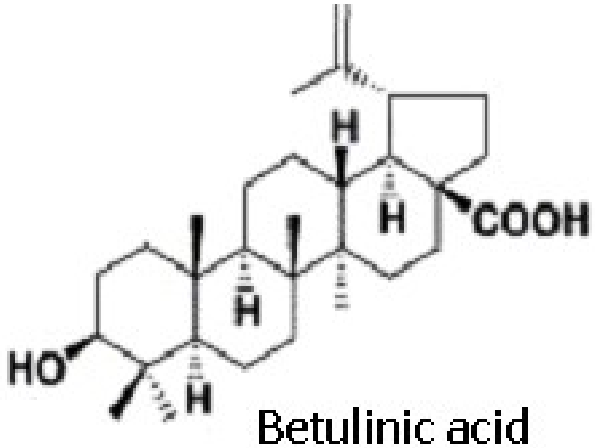
Even when new chemical structures are not found during drug discovery from medicinal plants, known compounds with new biological activity can provide important drug leads. Since the sequencing of the human genome, thousands of new molecular targets have been identified as important for various.

With the advent of high-throughput screening assays directed towards these targets, known compounds from medicinal plants may show promising and possibly selective activity. Several known compounds isolated from traditionally used medicinal plants have already been shown to act on newly validated molecular targets, as exemplified by indirubin, which selectively inhibits cyclin-dependent kinases and kamebakaurin, which has been shown to inhibit NF- kB. Other known compounds have also been shown to act on novel molecular targets, thus reviving interest in members of these frequently isolated plant compound classes. Three examples are cucurbitacin I, obtained from the National Cancer Institute (NCI) Diversity set of known compounds found to be highly selective in inhibiting the JAK/STAT 3 pathway in tumors with activated STAT 3, β-lapachone, which selectively kills cancer cells over normal cells through direct checkpoint activation during the cell cycle and betulinic acid, with selective melanoma cytotoxicity through the activation of p38[20&21].

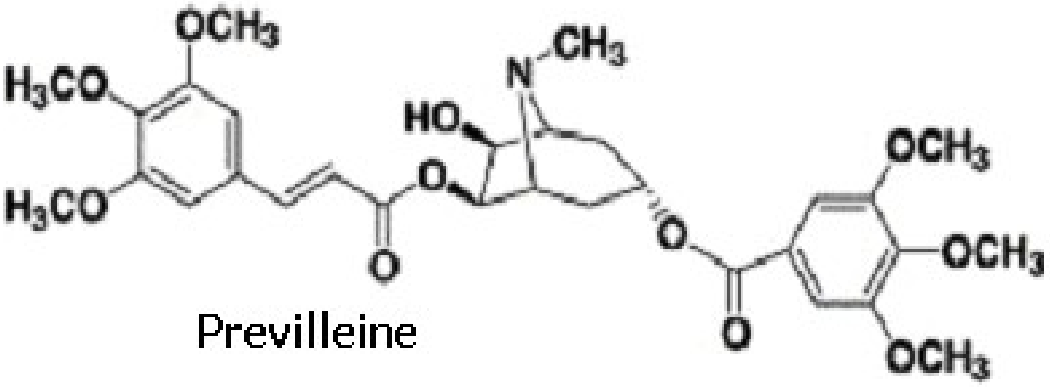
Some of the plant derived anticancer agents are under investigation for their effect on cancer cells. Some of them are as follows: Betulinic acid (9), a pentacyclic triterpene, is a common secondary metabolite of plants, primarily from Betula species (Betulaceae). The Betulinic acid isolated

from the ethyl acetate fraction of *Zisiphus mauritiana Linn* (Rhamnaceae) was found to have selective cytotoxicity against human melanoma cells. Previlleine A (10), along with eight other tropane alakaloids was isolated from the roots of Erythroxylum pervillei Bail (Erythroxylaceae) was found to be selectively cytotoxic against a multi-drug resistant (MDR) oral epidermoid cancer cell line (KB-V1) in the presence of anticancer agent vinblastine. Silvestrol (11) was first isolated from the fruits of Aglaia sylvestris (M Roemer) Merill (Meliaceae) (later re identified as Aglaia foveolata Pannel) was found to be cytotoxic against several human cancer cell lines [22].

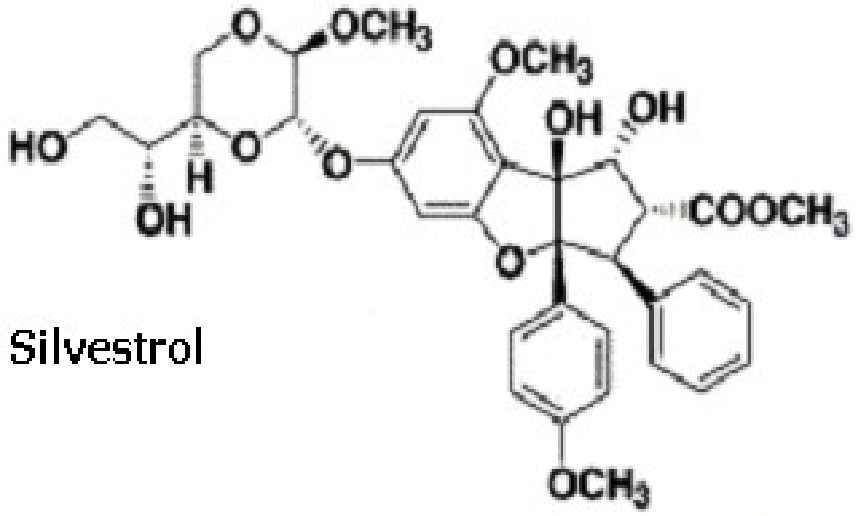
Fig. 02. **PLANT-DERIVED ANTICANCER AGENTS**



Betulinic acid



Previlleine

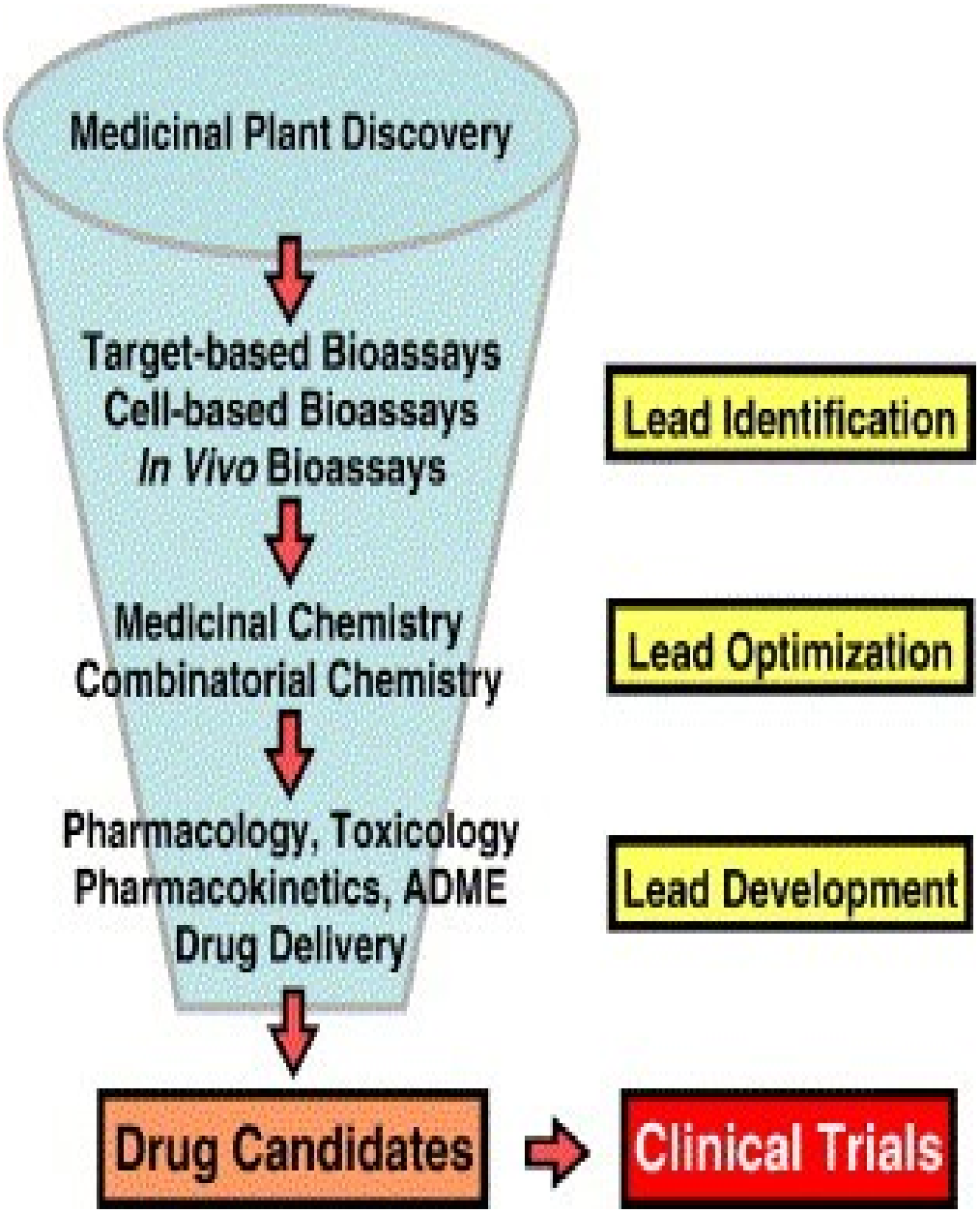


Silvestrol

Challenges in drug discovery from medicinal plants [23&24]

Despite the evident successes of drug discovery from medicinal plants, future endeavors face many challenges. Pharmacognosists, phytochemists and other natural product scientists will need to continuously improve the quality and quantity of compounds that enter the drug development phase to keep pace with other drug discovery efforts. The process of drug discovery has been estimated to take an average of 10 years upwards and cost more than 800 million dollars. Much of this time and money is spent on the numerous leads that are discarded during the drug discovery process. In fact, it has been estimated that only one in 5000 lead compounds will successfully advance through clinical trials and be approved for use. Lead identification is the first step in a lengthy drug development process (Fig). Lead optimization (involving medicinal and combinatorial chemistry), lead development (including pharmacology, toxicology, pharmcokinetics, ADME [absorption, distribution, metabolism and excretion] and drug delivery) and clinical trials take a considerable length of time.

Fig: 03 SCHEMATIC OF TYPICAL PLANT DRUG DISCOVERY AND DEVELOPMENT



Improving the speed of active compound isolation will necessitate the incorporation of new technologies. Although Nuclear Magenetic Resonance (NMR) and Mass Spectrometry (MS) are currently in wide use for compound identification, new methods of using NMR and MS could be applied to medicinal plant drug discovery to facilitate compound isolation. Also, the use of high-throughput X-ray crystallography could be applied to medicinal plant lead discovery. Compound development of drugs discovered from medicinal plants also faces unique challenges. Natural products are typically isolated in small quantities that are insufficient for lead optimization, lead development and clinical trials. Collaboratibg with synthetic and medicinal chemists is necessary to determine if synthesis or semi-synthesiis might be possible. Another technique to improve natural product compound development may involve the creation of natural product and natural-product-like libraries that combine the features of natural products with combinatorial chemistry.

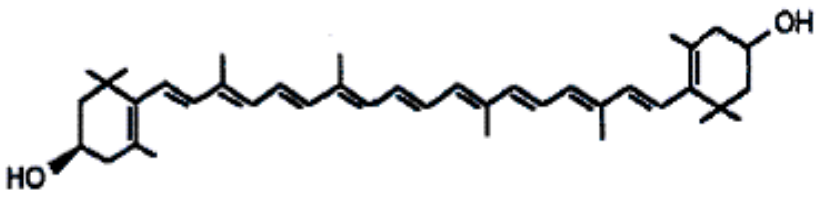
In conclusion, natural products discovered from medicinal plants (and derivatives thereof) have provided numerous clinically used medicines. Even with all the challenges facing drug discovery from medicinal plants, natural products isolated from medicinal plants can be predicted to remain an essential component in the search of new medicines.

REVIEW OF LITERATURE

2. WORKS DONE ON *DELONIX REGIA* (BOJ. EX HOOK.) RAF.

- R.K.Barua and A.B.Barua(1962) have isolated 3-hydroxy Retinene from Anthers of Delonix regia (Boj. ex Hook.) Raf flowers and also studied about the properties of 3-hydroxy retinene.Oxidation of Zeaxanthin with hydrogen peroxide in the presence of Osmium tetroxide results in the formation of 3-hydroxy retinene[30].

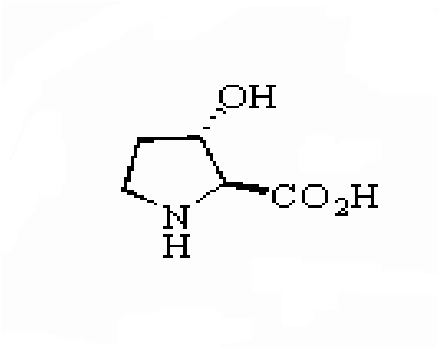
- Jungalwala F B et.al (1962) studied comparatively the amount and type of carotenoids present in various floral parts of *Delonix regia* (Boj. ex Hook.) Raf. The highest concentration of total carotenoids were found in the anthers of the flower. 90% of its found to be Zeaxanthin[31].



Zeaxanthin

- May-Lin-Sung et.al (1968) isolated trans -3-hydroxy Proline from seed of *Delonix regia (Boj. ex Hook.) Raf* and shown to inhibited the growth of mung bean seedlings. They also confirmed the presence of γ-Methylene Glutamine in seedlings also[32].

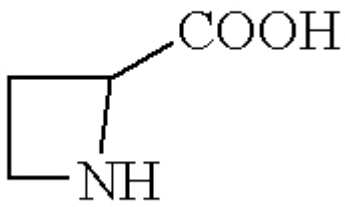
TRANS -3- HYDROXY -L- PROLINE



- L.Fowden et.al (1969) isolated Azetidine-2-carboxylic acid from the leaves of the legume *Delonix regia* (Boj. ex Hook.) Raf. The imino acid not be detected in dry seeds of the plant but it was produced rapidly during germination and it is present in all part of the plants [33].

- May-Lin-Sung et.al (1971) biosynthesized Imino acid and studied in *Delonix regia* (Boj. ex Hook.) Raf seedlings by labeled precursor feeding. α ,γ-Diaminobutyric acid was incorporated into Azetidine -2-carboxylic acid more efficienty than homoserine, methionine or aspartic acid. More radioactivity from proline was found in trans-3-hydroxy proline after 2 day's than after 4-day's metabolism, indicating a continuous turn over of the hydroxyl imino acid seedlings[34].

S (-) -2- AZETIDINE -2- CARBOXYLIC ACID



- V.P.Kapoor et.al (1972) isolated a galactomannan composed of (-) galactose and (-) mannose from the seed of *Delonix regia* (Boj. ex Hook.) Raf. It is established that galactomannan is a highly branched

polysaccharide consisting of the main chain of mannose united linked through β(1-4) and side chain of single galactose units linked through α (1-6) linkage[35].

- Leslie Fowden et.al (1973) studied comparatively the thermal stability and substrate binding constants of prolyl_t_RNA synthetase from Phaseolus aureus and *Delonix regia* (Boj. ex Hook.) Raf. The results are discussed in relation to the order of substrate specificity between the enzymes from Phaseolus aureus and *Delonix regia* (Boj. ex Hook.) Raf[36].

- Roger D Norris (1973) isolated enzyme Pro-t-RNA synthetase from Phaseouls aureus and Delonix regia. Pro-t-RNA synthetase was photo inactivated in the presence of methyelne blue or Rose Bengal. Pro and several imino acid analogues protected the enzyme against Dye –mediated photo inactivation but ATP was ineffective[37].

- D. Mukherjee et.al (1975) demonstated Proline and hydroxyl proline biosynthesis from the floral parts and buds of *Delonix regia* (Boj. ex Hook.) Raf. The identified phytoconstituents such as α-ketoglutaric acid, oxalo acetic acid , pruvic acid and glyoxylic acid from floral parts and buds. They also reported on the calyx and androecium accumalate glyoxlic acid in amounts greater than those reported from other plants[38].

- Roger D Norris et .al (1975) obtained partially purified preparation of Phe and Tyr –t-RNA synthetases from seed or seedlings of Phaseolus

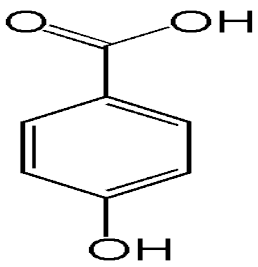
aureus, Further they had demonstrated *Delonix regia* (Boj. ex Hook.) Raf and *Caesalpinia tinctoria* ability of a variety of structural analogues of Phe or Tyr to act as alternative substrates or inhibitors[39].

- Nabiel.A.M.et al (1976) reported Anthocyanins of some leguminosae flowers and their effect on colour varation *Bauhinia variegate*, *Xassia nodosa* and *Delonix regia* (Boj. ex Hook.) Raf. Flavonoids :Anthocyanins were screened in the above plant for its colour variation[40].
- Szymanovicz G et.al (1978) newly synthesized 3-hydroxy proline. That is a new method of preparation and some properties of 3-hydroxy proline from seed extract of *Delonix regia* (Boj. ex Hook.) Raf. The hydrolyzate is fractionated sequentially on Resin and sephadex. Purification and characterization of 3-hydroxy proline clearly separated from 4-hydroxy proline was carried out by means of TLC chromatography and high voltage paper electrophoresis[41].

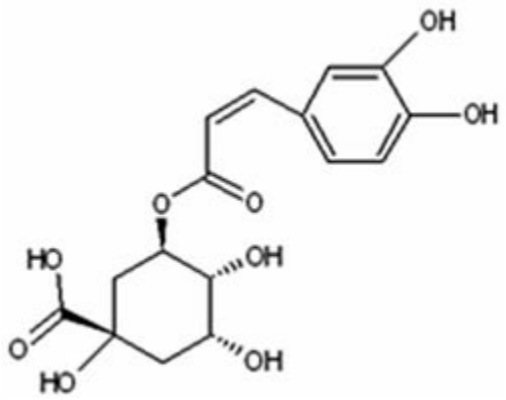
- Mendes NM et. al (1986) screened Molluscicide activity of aqueous (macerated and boilrd) hexanic and ethylic extracts of *Aristolochia brasiliensis, Ceasalpinia peltophoroides, Delonix regia* (Boj. ex Hook.) Raf. The most active of the extracts studied was *Delonix regia* (Boj. ex Hook.) *Raf* flowers (flamboyant) ethylnic extracts which presented mollusicidal activity on adult snails at 20ppm[42].

- Saxena.S.C et.al (1986) evaluated *Delonix regia* (Boj. ex Hook.) Raf for disruptor of insect growth and development. It's a preliminary laboratory evaluation of an extract of leaves of *Delonix regia* (Boj. ex Hook.) Raf disruptor of insect growth and development[43].
- Marfo.E.K. (1989) evaluated chemical and nutritional properties of Flamboyant beans (Delonix regia) *Delonix regia* (Boj. ex Hook.) Raf[44].
- Kpikpi.W.M (1992) RATD tried *Musanga cropioides* and *Delonix* regia (Boj. ex Hook.) Raf as papermaking hardwoods[45].
- Channg-Hung Chou (1992) bioassayed a series of aqueous extracts of leaves, flowers and twigs of *Delonix regia* (Boj. ex Hook.) Raf against three species to determine their phytotoxicity and the results showed highest inhibition in the flowers. By means of TLC, HPLC and Paper chromatography and UV-Visible spectrometry the responsible phytotoxins present in leaves, flowers and twigs of *Delonix regia* (Boj. ex Hook.) Raf were identified as 4-hydroxy benzoic, chlorogenic .etc [46]

4 – HYDROXY BENZOIC ACID



CHLOROGENIC ACID



- Chou .C.H.et.al (1993) studied about the allelopathic substances and its interaction of *Delonix regia* (Boj. ex Hook.) Raf with other species of *Delonix* [47].

- Dutta et.al (1998) studied invitroly the aqueous extracts of plants such as *Terminalia chebula, Punica granatum, Delonix regia* (Boj. ex Hook.) Raf and *Emblica officinals* for Dermatophytes[48].
- Enikuomehin OA. et.al (1998) evaluated eleven ash samples from organs of nine tropical plants for their abilites to inhibit mycelial growth and sclerotial germination of a Nigerian isolate of *sclerotium rolfsii* on agar and in the soil. Ash sample from *Delonix regia* (Boj. ex Hook.) Raf stem wood, *Magifera in*dica leaf and *Vernonia amygdalina* leaf were most effective as each totally inhibited mycelial growth of *Sclerotium rolfsii* in vitro[49].
- Polikarpov I.et.al (1999) purified, crystallized studied preliminary crystallographic study of a Kunitz –type trypsin inhibitor from *Delonix regia* (Boj. ex Hook.) Raf seeds. The Kunitz-type trypsin inhibitor from seeds of Flambyoant has been purified to homogeneity and plate like crystals suitable for X-ray analysis have been grown by the hanging-drop method, the structure has been solved by molecular replacement using the known structure of Trypsin inhibitors from *Erythrina Caffra* seeds, Soya beans as search models[50].

- Muruganaandan.et.al (2001) screened anti-inflammatory and analgesic activities of some Medicinal plants. The extracts of some medicinal plants were used at the dose rate of 300Kg,p.o. Aspirin (300mg/Kg,p.o) was

employed as reference drug. Significant anti-inflammatory activity was observed with *Delonix regia* (Boj. ex Hook.) Raf bark. Pongamia Pinnata seeds, *Psidium guavajava* leaves and *Aegle marmelos* bark[51].

- Srinivasajn,.K.et.al (2001) evaluated seventy percent ethanolic extracts (300mg/Kg p.o.) of *Delonix regia* (Boj. ex Hook.) Raf (Bark and Flowers), *Psidium guavajava* leaves, *Aegle marmelos* (Bark) exhibited significant anti-inflammatory activities in rats. However *Butea frondosa* (Flower) *Pinus longifolia* (Leaves) *Eugeia jambolana* didn't exhibit significant activity. *Pongamia pinnata* (Seeds) and *Delonix regia* (Boj. ex Hook.) Raf (Bark and Flowers) exhibited significant ANALGESIC Activity[52].
- Oliva ML .et al. (2001) isolated a serine protienase inhibitor and purified from *Delonix regia* (Boj. ex Hook.) Raf seeds a Leguminosae tree of the Ceasalpinioidae subflamily. The inhibitor named DrTI, inactivated trypsin and Human plasma Kallikrein with K(i) values 2.19x108M nd 5.25nM,respectivey. The primary sequence of the inhibitor was determined by Edman degradation and 185 amino acids showed that it belongs to the Kunitz type inhibitor family. However its reactive site

didn't contain contain Argine or Lysine at the putative reactive position or it was displaced when compared to other Kunitz type inhibitors[53].

- Pando SC et.al (2002) characterized a lectin from the *Delonix regia* (Boj. ex Hook.) Raf seeds which was purified by gel filtration on Sephadex G-100 followed by Ion exchange chromatography on Diethyl amino –ethyl sepharose and reverse –phase HPLC on a C 18 column. Haemagglutinating activity was monitored using rat erythrocytes. DRL showed no specificity for human erythrocytes of ABO blood group[54].
- Sampaio et el.(2002) determined the Primary sequence of a Kunitz Inhibitor which is isolated from *Delonix regia* (Boj. ex Hook.) Raf seeds [55].
- Ankrah NA.et.al (2003) evaluated the efficacy and safety of a herbal medicine used for the treatment of malaria. The resistance of *Plasmodium falciparum* to Choloroquine has been reported in several countries. This has led to renewed interest in the development of herbal medicines that have the potential to treat malaria with little or no side effects. This study obtained a preliminary information on the safety and effectiveness of *Jatropha curcas, Gossypium hirsutum,Physalis angulata* and *Delonix regia* (Boj. ex Hook.) Raf used in treating malaria[56].

- Krauchenco S.et al.(2003) studied the three dimensional structure of a novel Kunitz (STI) family member, an inhibitor purified from *Delonix regia* (Boj. ex Hook.) Raf seeds (DrTI) was solved by molecular replacement method and refined respectively. The structure has a classical beta-three fold, however differently from canonical grounds of such specificity are discussed[57].
- Ahmad I et al (2003) screened for broad spectrum anti-bacterial, anti-fungal activities and potency of crude alcoholic extract and fractions of *Delonix regia* (Boj. ex Hook.) *Raf*. 70% Ethanolic crude extract was

further fractionated with Petroleum ether, benzene, acetone, ethyl acetate and methanol. Anti-microbial activity of crude extract and fractions was tested against nine bacteria six filamentous Fungi and a Yeast[58].

- Seetharam et.al (2003) screened anti-microbial and analgesic activity of *Delonix regia* (Boj. ex Hook.) Raf and *Delonix elata* gamble Raf. Ethanolic extract of (delonix regia) and (Delonix elata) have shown good anti-microbial activity (40 mg/10ml) and analgesic activity at the dose of 200mg/Kg b.w[59]
- Mahmood .Z et .al (2003) evaluated Antioxidant properties of extracts and fractions of chichory, tulsi and gulmohar. Crude alcoholic extract of *Ocimum sanctum* was fractionated into ethyl acetate and methanol.

Similarly [*Cinchorium intybus*] extracts in benzene and acetone, alcoholic extract of *Delonix regia* (Boj. ex Hook.) Raf were used. AlphaTocopherol and Butylated hydroxyl toluene were used as standard antioxidants[60].

- Venkateswara Rao et.al (2004) screened the various fractions of crude methanolic extracts and alkaloidal fractions of *Delonix regia* (Boj. ex Hook.) Raf (flowers) were tested for their anti-bacterial activity . The water soluble fractions of methanolic extract was found to be effective against all the tested organisms[61].
- Aqil. F et al(2004) worked on Delonix regia (Boj. ex Hook.) Raf (Flowers) Camellia sinesis (Leaves) Holarrhena antidysentrica (Bark) Lawsonia inermis (Leaves) Punica granatum (Rind), Terminalia chebula (fruits), Terminalia bellerica (fruits). Crude extracts of above showed broad spectrum antibacterial activity against all MRSA and a methicillin sensitive strains with inhibition zone size of 11mm to 27mm[62].
- Seneviratne.G et al (2004) screened the quality of different Mulch materials and their decomposition and nitrogen release under low moisture regimens. Six leguminosal leaves including *Delonix regia* (Boj. ex Hook.) Raf leaves with a high phenolic and carbon content which were subjected

to leaching losses of these fractions underwent a change in their N dynamics from net immobilization to mineralizations[63].

- Al.Bahry et al(2005) reported *Ganoderma colossum* on *Ficus altissima* and *Delonix regia* (Boj. ex Hook.) Raf[64].
- Oswasis et al (2005) investigated the ethanolic extracts and some fractions from 10 Indian medicinal plants including *Delonix regia* (Boj. ex Hook.) Raf, known for antibacterial activity for their ability to inhibit clinical isolates of β-lactamase producing methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *S.aureus* (MSSA). Synergistic interaction of plant extracts with certain antibiotics was also evaluated[65].
- Koster et al(2005) compared the various method of diffusive gels in thin films with conventional extraction techniques for evaluating Zinc accumulation in plants and isopods of *Delonix regia* (Boj. ex Hook.) Raf [66].
- Jigna Parekh et al (2005) screened twelve medicinal plants namely *Abrus precatorius, Caesalpinia pulcherrima, Cardiospermum halicacabum, Casuarina equisetifolia, Cynodox dactylon, Delonix regia* (Boj. ex Hook.) Raf, *Euphorbia hirta, Euphorbia tirucallic, Ficus*

benhalensis, Gmelina asiatica, Santalum album and *Tecomella undulate* for potential antibacterial activity against 5 medicinally important bacterial strains. From the screening experiment *Caesalpinia* showed the best antibacterial activity[67].

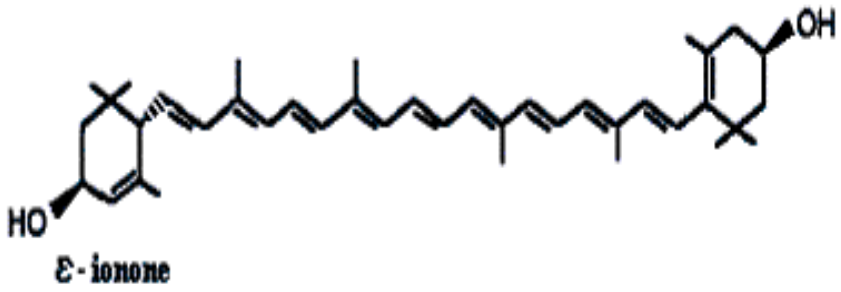
- Farrukh Aqil et al (2006) screened the methanolic extracts of 12 traditionally used Indian medicinal plants including *Delonix regia (Boj. ex Hook.) Raf* for their antioxidants and free radical scavenging properties using α-Tocopherol and Butylated Hydroxy Toluene(BHT) as standard antioxidants. A fair correlation between antioxidant free radical scavenging activity and phenolic content was observed among 9 plants. The tested plant extracts showed promising antioxidant and free radical scavenging activity thus justifying their traditional use[68].
- Oluwasola Agbede.J et al (2006) characterised the leaf meals. Protein concentrates and residues from some trophical leguminous plants like Butterfly pea (*Cetrosema pubescens*),Devil Bean (*Mucuna pruiens*) Flamboyant flowers (*Delonix* regia (Boj. ex Hook.) Raf, *Bauhinia tomentosa,Wart Wattle* (*Acacia auriculiformis*) were analysed for their nutrient and anti-nutritional content. The leaf protein concentrates(LPCs) were produced from the leaves by fractionation and characterized along with the fibrous residues[69].

- Sumitra, Chandra et al (2006) screened the in vitro anti-microbial activity and Phytochemical analysis of some medicinal plants including *Delonix regia* (Boj. ex Hook.) Raf[70].
- Pandeya .S.C et al (2007) studied the genetic diversity in some perennial plant species within short distances. *Delonix regia* (Boj. ex Hook.) Raf depicted highest similarity between Lucknow and Agra. *Calotropis procera* of Lucknow location was more closer to Gwalior than Agra. The results confirmed genetic diversity in the species as a means of adaptation to differing climo-edaphic variables[71].
- Ahmad.I et al (2007) screened 66 ethanolic plant extracts including *Delonix regia* (Boj. ex Hook.) Raf against 9 different bacteria. Of these 39 extracts demonstrated activity against 6 or more test bacteria. Twelve extracts showing broad spectrum activity was tested against Multidrug-Resistant(MDR) bacteria,Methicillin-resistant. Staphylococcus aureus(MRSA) and extended spectrum β-lactamases producing Enteric bacteria[72].
- Hung.C.H et al (2007) designed degenerate primers based on all possible sequences of the N-terminal and C-terminal regions of *Delonix regia* (Boj. ex Hook.) Raf trypsin inhibitor. Genomic and cDNA cloning,characterization of *Delonix regia* (Boj. ex Hook.) Raf trypsin

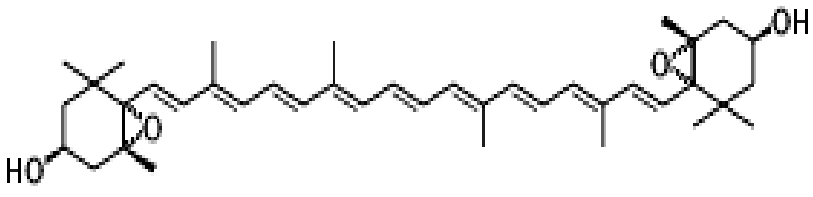
inhibitor CDrTI gene and expression of DrTI in E.coli. Both the recombinant *Delonix regia* (Boj. ex Hook.) Raf trypsin inhibitor (DrTI) and glutathione-S-transferase(GST). DrTI fusion protein exhibited a strong identical inhibitory effect on Trypsin activity[73].

- Anitha.K et al (2007) developed multiple natural dyes from flower parts of Gulmohar which contains flavonoids such as Leuco anthocyanin; caratenoids such as lutein, zeaxanthin, violoxanthin, neoxanthin, auroxanthin, 5,6-monoepoxylutein, antheraxanthin and flavoxanthin which are responsible for dyeing[74].

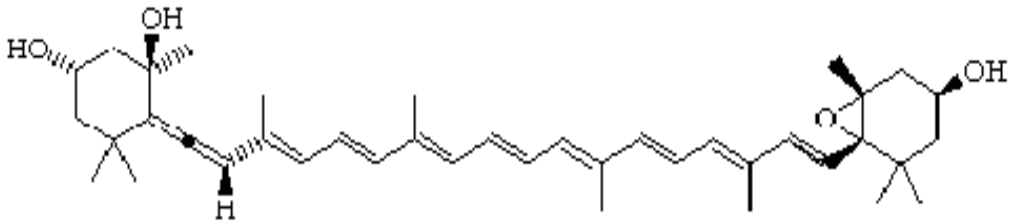
LUTEIN



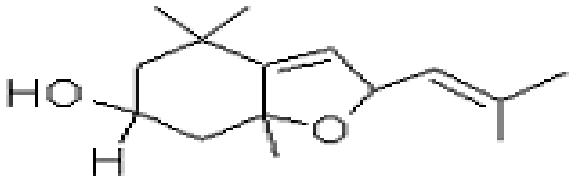
VIOLOXANTHIN



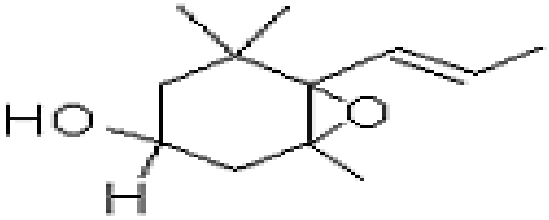
NEOXANTHIN



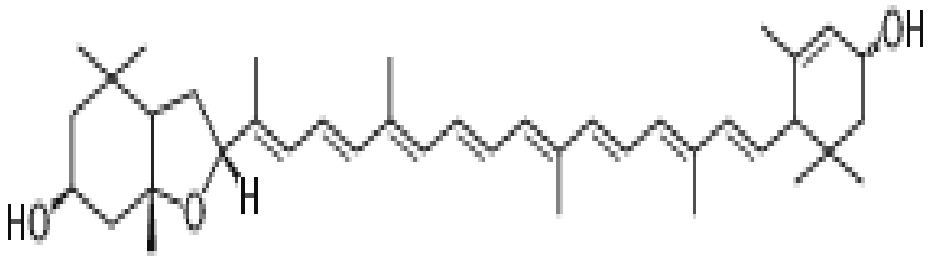
AUROXANTHIN



ANTHEROXANTHIN



FLAVOXANTHIN



- Abiola.O.K et al (2007) investigated the inhibitive effect of *Delonix regia* (Boj. ex Hook.) Raf extracts to reduce the corrosion rate of aluminium in acidic media. The study was a trial to find a low cost and environmentally safe inhibitor to reduce the corrosion rate of aluminium[(75)].

3. PHARMACOLOGICAL ACTIVITIES

4. 1. OXIDATIVE STRESS [100-102]

Oxidative stress caused by free radicals has become an area of interest in understanding the process of human disease. However, interpretation of the term “oxidative stress” itself is often confused and the processes involved poorly understood. The term oxidative stress has rarely been defined in a universally accepted way. One accepted definition by Sies in 1991 is “ a disturbance in the pro-oxidant - antioxidant balance in favour of the former, leading to potential damage.” For a disturbance in this balance to occur, it follows that one or both of the following scenarios must be present.

1) A reduction in antioxidant.

2) An increase in reactive species.

1.1.Oxidative Stress and Diseases

It is quite certain that oxidative stress is the cause of a few human diseases. For example, formation of $OH^{\cdot}$ by ionising radiation is thought to be responsible for diseases related to exposure to ionising radiation. The results of exposure to high O_2 concentrations and the various congenital and malnutritional causes of depleted antioxidants may also be attributed to oxidative stress along with a few other diseases.

However, tissue injury itself has been shown to be a major cause of oxidative stress via many pathways including the activation of phagocytes, release of metal ions and excess electron leakage from the electron transport chain. The current consensus suggests that oxidative stress is a consequence of tissue damage in many diseases rather than the major cause. The extent of the role played by oxidative stress in the pathogenesis of disease is thought to vary in different diseases. However, although oxidative stress may seldom be a primary cause of disease, its importance should not be dismissed as a potential target for therapeutic treatment.

1.2. Free Radical

A free radical is defined as “any species capable of independent existence that contains one or more unpaired electrons”. Generally, free radicals are more reactive than non-radicals (although an oxygen molecule O_2 is classed as a free radical and is not particularly reactive) and will react with them to produce new free radicals in a chain reaction. It is these chain reactions that can lead to damage to molecules in the body. A reaction between two free radicals will result in the pairing of their unpaired electrons and therefore non-radicals are formed.

1.2.1. Free Radical Mechanism

Free radicals attack three main cellular components.

*** Lipids**: Peroxidation of lipids in cell membranes can damage cell membranes by disrupting fluidity and permeability. Lipid peroxidation can also adversely affect the function of membrane bound proteins such as enzymes and receptors.

*** Proteins**: Direct damage to proteins can be caused by free radicals. This can affect many kinds of protein, interfering with enzyme activity and the function of structural proteins.

- **DNA**: Fragmentation of DNA caused by free radical attack causes activation of the poly(ADP-ribose) synthetase enzyme. This splits NAD+ to aid the repair of DNA. However, if the damage is extensive, NAD+ levels may become depleted to the extent that the cell may no longer be able to function and will die.

The precise site of tissue damage by free radicals is dependent on the tissue and the reactive species involved. The overall damage caused by oxidative stress is often an accumulation of damage to many sites. Extensive damage can lead to death of the cell; this may be by necrosis or apoptosis depending on the type of cellular damage.

1.2.2. General Features of free radical reaction[103]

Free radical reactions take three distinct identifiable steps, such as

01. **Initiation step:** Formation of radicals.

02. **Propagation step:** It is the heart of a free radical reaction. In this step, the required free radical is regenerated repeatedly, which would take the reaction to completion.

03. **Termination step:** Destruction of radicals.

Fig : 4 ***Free Radical reactions step wise***

I. Initiation Step

$$RH \longrightarrow R^* + H$$

Organic Compound Free Radical

II. Propagation Step

$$R^* + O_2 \longrightarrow RO_2^*$$

$$RO_2^* + RH \longrightarrow RO^* + OH^* + R^*$$

Hydroxy radical

III. Termination Step

$$R^* + H \longrightarrow RH + I^*$$

Stable free radical

$$RO_2^* + X \longrightarrow \text{Inactive Products}$$

(Termination by inhibitor)

Were X is a Chain Inhibitors.

1.2.3. Sources of Free radicals [104]

Two types of sources are:

1. Exogenous free radicals

2. Endogenous free radicals.

1.2.3.1. Exogenous sources of free radicals

Exogenous sources of free radicals are automobile exhaust fumes, UV radiation, interaction with chemicals, smoking of cigarettes, cigars, beedies, etc., (one puff of cigarette smoke is estimated to contain 10^{14} free radicals with > 4000 compound, including NO and NO_2*), burning of organic matter during cooking, forest fires, etc, volcanic activities, radioactive decay-α, β and γ radiation, lightening particularly oxides of nitrogen, byproduct of oxygen metabolism (illness causes the body to produce greater amounts of harmful radicals than in healthy condition), industrial effluents, excess

chemicals, alcoholic intake, certain drugs, asbestos, certain pesticides, some metal ions, fungal toxins etc., inflict oxidative stress.

1.2.3.2. Endogenous sources of free radical

They include cyclooxygenation, lipooxygenation, lipid peroxidation, neutrophils stimulated by exposure to microbes, reperfusion of ischemic organs, mechanism of xenobiotics and UV and ionizing radiation damage.

1.2.4. Types of free radicals

The most important free radicals in the body are the derivatives of oxygen, better known as reactive oxygen species.

Table : 27 **Free radicals and their structure**

S.No	Types of free radicals	Structure
01	Superoxide anion	O_2*
02	Hydroxyl radical	OH*
03	Lipid peroxyl radical	LO_2*
04	Singlet oxygen	O*
05	Hydrogen peroxide	H_2O_2*
06	Hypochlorous acid	HOCl*
07	Peroxy nitrate	ONOO*
Other common free radicals		
08	Hydroperoxyl	HO_2*
09	Peroxyl	RO_2*
10	Alkoxyl	RO*
11	Hydrogen centered radicals	(H*)
12	Carbon centered radicals	(CCl_3*)
13	Sulfur centered radicals	(RS*)

1.2.4.1. Superoxide anions (O_2*)[105]

Superoxide anion is the first reduction product of oxygen. It is a base with the equilibrium with its conjugate acid, the hydroperoxyl radical HOO*. The formation of superoxide takes place spontaneously, especially in the electron rich aerobic environment in vicinity of the inner mitochondrial membrane with the respiratory chain. Superoxide is also produced endogeneously by flavonoenzymes e.g., lipoxygenase and cyclooxygenase. The nicotinamide adenine dinucletide phosphate (reduced form) (NADPH) dependent oxidase of phagocytic cells, a membrane associated enzyme complex, constiitues and example of deliberate high level O_2* production.

Two molecules of superoxide rapidly dismutase to hydrogen peroxide and molecular oxygen and this reaction is further accelerated by superoxide dismutase (SOD)

1.2.4.2. Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is the most stable reactive oxygen species. H_2O_2 is the primary product of the reduction of oxygen by various oxidase such as xanthine oxidases, uricase, D-amino acid oxidase and α-hydroxy acid oxidase localized in peroxisome. Research shows that the H_2O_2 is the most effective species for cellular injury. It plays an important role in the production of more ROS molecules including HOCl (Hypochlorous acid) by the action of myeloperoxidase an enzyme present in the phagosomes of the neutrophils and most importantly, formation of OH* via oxidation of transition metals.

$$\mathbf{H^+ + Cl^- + H_2O_2 \longrightarrow HOCl + H_2O}$$

The most important function of H_2O_2 is its role as an intracellular signaling molecule.

H_2O_2 once produced by the above mechanism is removed by atleast three antioxidant enzyme systems, namely catalases, glutathione peroxidases and peroxidredoxins.

1.2.4.3. Hydroxy radicals (OH*) [106]

Hydroxy radical is highly reactive. It may react with any molecule present in the cells. For this reason it is short lived. The life span of OH* at 37℃ is 10^{-9} sec. The hydroxy radical is formed from hydrogen peroxide in a reaction catalyzed by metal ions (Fe^{+} or Cu^{+}), often bound in complex with different proteins or other molecules. This is known as Fenton reaction.

$$\mathbf{H_2O_2 + Cu^{+} / Fe^{2+} \longrightarrow OH^{*} + OH^{-} + Cu^{+}/Fe^{3+} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)}$$

Superoxide also plays an important role in connection with reaction 1 by recycling the metal ions.

$$\mathbf{Cu^{2+} / Fe^{3+} + O_2^{*} \longrightarrow Cu^{+}/Fe^{2+} + O_2 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)}$$

The sum of reaction 1 and 2 is the Haber-Weiss reaction; Transition metals thus play an important role in the formation of hydroxy radicals. Transition metals may be released from proteins such as ferritin and the (4Fe- 4S)

center of different dehydrases by reactions with O_2*. This mechanism, specific for living cells, has been called the *invivo* Haber-weiss reaction. Lipid is very sensitive to OH* attack and initiates LPO (Lipid Peroxidation). The hydroxy radical is responsible for DNA damage and LPO.

1.2.4.4. Malonyldialdehyde (MDA)

Malonyldialdehyde is the major reactive aldehyde resulting from the peroxidation of biological membrane poly unsaturated fatty acid (PUFA), a secondary product of LPO is used an indicator of tissue damage by a series of chain reaction. MDA is also a by product of prostaglandin biosynthesis. MDA is mutagenic and genotoxic agent that contribute to the development of human cancer.

1.2.4.5. Nitric Oxide (NO*)

Nitric oxide is an inorganic free radical gas. It is synthesized by nitric oxide synthesis located in various tissues and plays active role in free radical tumour biology. NO is synthesized enzymatically from L-arginine by NO synthase.

$$\textit{L-arginine} + O_2 + \textit{NADPH} \longrightarrow \textit{L –citrulline} + \textit{NO} + \textit{NADP}^+$$

Nitric oxide is another free radical that has an important biological role. NO* produced in the body relaxes muscles in blood vessels and lowers blood pressure. Many blood pressure lowering drugs e.g., Nitroglycerine, amyl nitrite. But, excess NO* produced in cases of severe infection can be harmful. Unlike HO* or O2*, NO* is a much slower reacting radical and it combines with other free radical and inhibits further reaction or generate more reactive product.

1.2.5. Chemistry of free radical generation [107]

Free radicals can be generated both *in vivo* and *In vitro* by one of the following mechanisms.

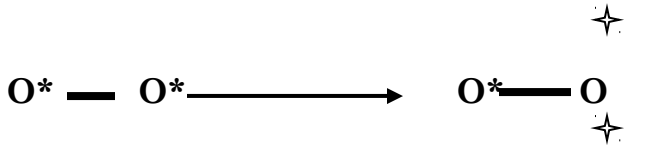
- ❖ Homolytic cleavage of covalent bond in which a normal molecule fragments into two, each fragment retaining one of the paired electrons. Homolytic cleavage occurs less commonly in biological systems, as it requires high-energy input from UV light, heat or ionizing reaction.

- ❖ Loss of single electron from a normal molecule.
- ❖ Addition of an electron to normal molecule.

1.2.6. Mechanism of action of free radicals or ROS formation [108]

Oxygen in the atmosphere has two unpaired electrons and these unpaired electrons have parallel spins. Oxygen is usually non reactive to organic molecules that have paired electrons with opposite spins. This oxygen is considered to be in a ground (triplet or inactive) state and is activated to a singlet (active) state by two different mechanisms.

a) Absorption of sufficient energy to reverse the spin on one of the unpaired electrons

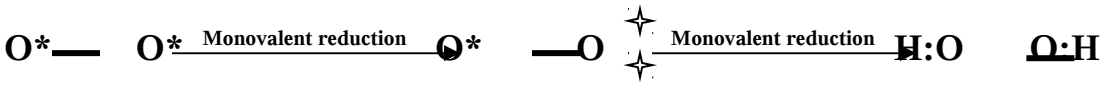


Triplet Oxygen↑↓ Singlet Oxygen↑↓

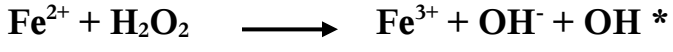
Ground State *Highly reactive*

b) Monovalent reduction (accept a single electron)

Superoxide is formed in the firs monovalent reduction reaction, which undergoes further reduction to form H_2O_2. H_2O_2 is further gets reduced to hydroxyl radicals in the presence of ferrous salts (Fe2+). This reaction was first described by Fenton and later developed by Haber and Weiss.



Fenton Reaction



Haber Weiss Reaction

$$H_2O_2 + OH^- \longrightarrow H_2O + O_2 + H^-$$

$$H_2O_2 + O_2{}^{*-} \longrightarrow O_2 + OH^- + OH^*$$

1.2.7. Diseases caused by the free radicals[109-111]

The free radicals are generated during the normal metabolic reaction in the body. Free radicals are very unstable and react quickly with other compounds, trying to capture the needed electron to gain stability.

Some free radicals arise normally during metabolism sometimes the body's immune system cells purposefully create free radicals to neutralize virus and bacteria. However environmental factors such as pollution, cigarette smoke and herbicide can also produce free radicals. Normally the body can handle free radicals but if antioxidants are unavailable or if the free radical production is excess then damage can occur.

The formation of free radicals and the occurrence of oxidative stress is a common component of parkinsons disease have reduced glutathione levels and free radical damage is found in the form of increased lipid peroxidation and oxidation of DNA bases.

The disease occur due to free radicals area as follows:

- **Cancer and other Malignancies**
- **Lipid peroxidation and Artherosclerosis**
- **. Lung disease**
- **Infertility**
- **Arthiritis**
- **Diabetes Mellitus**
- **Muscle Damage**
- **Inflammation**

1.3. Antioxidants[113]

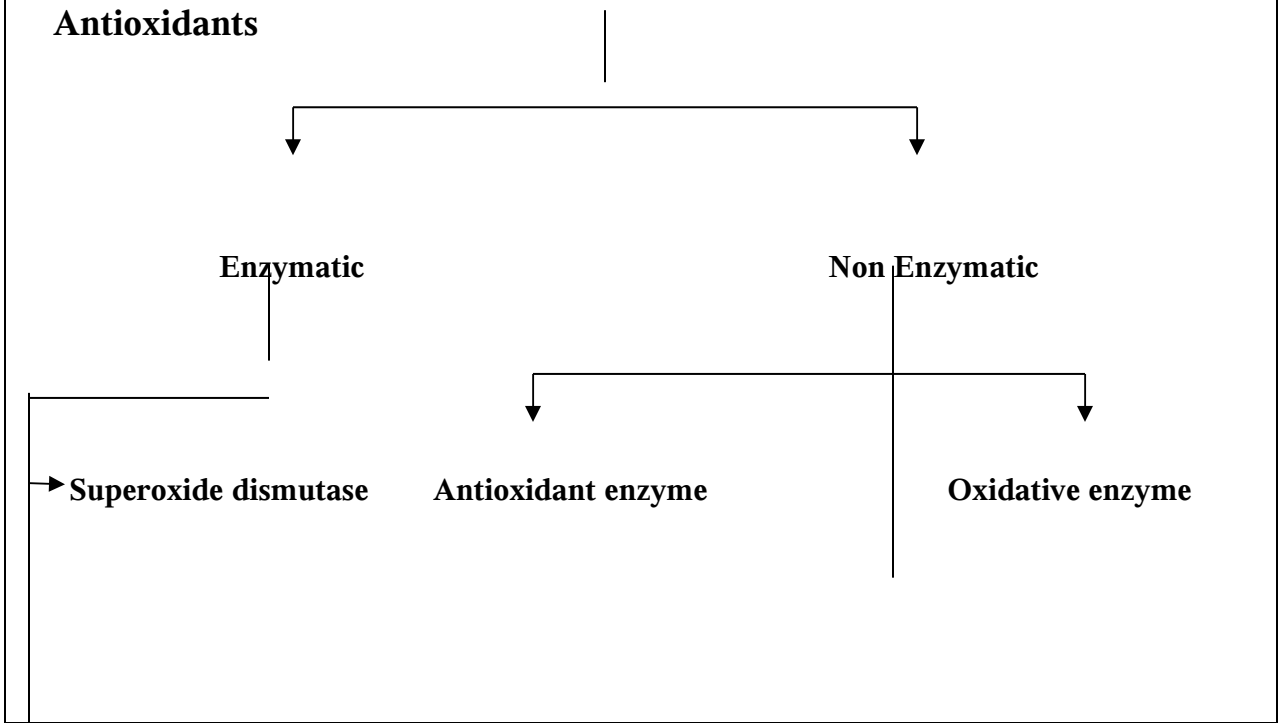
Antioxidants have been defined “as any substance which delays or inhibits oxidative damage to a target molecule.” In general, an antioxidant in the body may work in one of five ways.

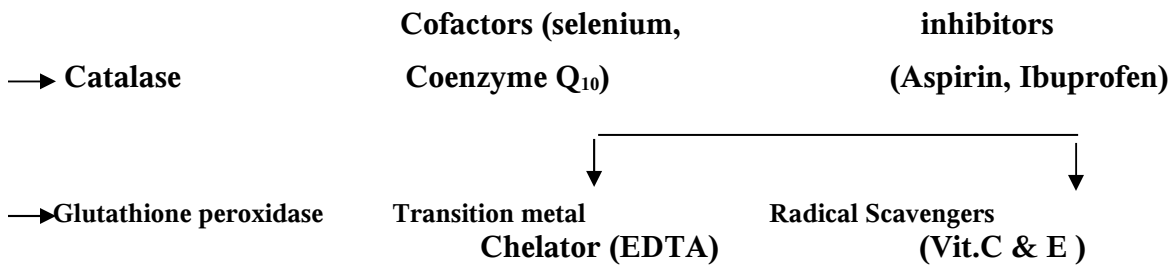
- Replacing damaged “target molecules”
- Keeping formation of reactive species to a minimum
- Repairing damaged “target molecules”
- Binding metal ions required for formation of highly reactive species (such as OH.)
- Scavenging reactive species either by using enzymes or directly by reaction whereby the antioxidant itself would be used up.

1.3.1. Antioxidants system/System of antioxidant.

The body had developed several endogenous antioxidant systems to deal with the production of reactive oxygen intermediates (ROI). These systems can be divided into:

Fig: 5 **Classification of Antioxidants**





Enzymatic antioxidants include superoxide dismutase (SOD) which catalyses the conversion of O_2^- to H_2O_2 and H_2O; catalase which then converts H_2O_2 to H_2O and O_2, and glutathione peroxidase which reduces H_2O_2 to H_2O.

Both Vit C and GSH have been implicated in the recycling of α -t-cpherol radicals. In addition, the trace elements such as selenium, manganese, copper and Zinc also play important roles as nutritional antioxidant cofactors. Selenium is a cofactor for the enzyme glutathione peroxidase and manganese, copper and Zinc are cofactors for SOD.

Zinc also acts to stabilize the cellular metallothionein pool, which has direct free radical quenching ability. Glutathione is recycled by nicotinamide adenine dinucleotide phosphate (reduced form), which is facilitiated by glutathione reductase.

1.3.2. Enzymatic Antioxidants

1.3.2.1. Superoxide dismutase (SOD) [114&115]

SOD is an endogenously produced intracellular enzyme present essentially in every cell in the body. Cellular SOD is actually represented by a group of metalloenzymes with various prosthetic groups. The prevalent enzyme is cuprozinc (Cu Zn) SOD which is a stable dimeric protein (32,000).

SOD appears in 3 forms according to the catalytic metal present in the active site.

i. Cu-Zn Sod in the cytoplasm and contains copper and zinc as metal cofactors.
ii. Mn-SOD in the mitochondria and contain Mn.
iii. Extracellular SOD recently has been described contains copper (CU-SOD).

$$2O_2^- \; 2H^+ + \; SOD \longrightarrow \; H_2O_2 + O_2$$

SOD scavenges both intracellular and extra cellular superoxide radical and prevents the lipid peroxidation of plasma membrane. However it should be

conjugated with catalase or GPx to prevent the action of H_2O_2, which promotes the formation of hydroxyl radicals. SOD also prevents hyper activation and capacitation induced by superoxide radicals.

1.3.2. 2. Glutathione peroxidase (GPx) reductase/enzyme (GRD) [116&118]

It is the tetrameric protein 85,000 D and has 4 atoms of Selenium (Se) bound as seleno-cysteine moieties that confer the catalytic activity. One of the essential requirement is glutathione as a cosubstrate.

Glutathhione peroxidase reduces H_2O_2 to H_2O by oxidizing glutathione (GSH) reproduction of the oxidized form of glutathione (GSSH) and then catalyzed by glutathione reductase. These enzymes also requires trace metal factors for maximal efficiency, including selenium for glutathione peroxidase; copper, zinc or manganese for SOD; and iron for catalase.

H_2O_2 + 2 GSH $\longrightarrow$ GSSG + 2 H2O (Glutathione Peroxidase)

GSSG + NADPH + H^+ $\longrightarrow$ 2 GSH + $NADP^+$ (Glutathione Reductase)

GSH = Reduced glutathione. GSSG = Oxidised glutathione.

1.3.2.3. Catalase

Catalase is a protein enzyme present in most aerobic cells in animal tissues and also present in all body organs being especially concentrated in liver and erythrocytes, while brain, heart, skeletal muscle contains only low amounts.

Catalase and glutathione peroxidase decomposes hydrogen peroxide to water and molecular oxygen.

$$2H_2O_2 \xrightarrow{\text{Catalase}} H_2O + \tfrac{1}{2} O_2$$

1.3.3. Non enzymatic antioxidants

1.3.3.1. α -Tocopherol (Vitamin E)

Vitamin E is the major lipid soluble antioxidant found in cells. Tocopherols are present in oils, nuts, seeds, wheat germ and grains. In nature, 8 substances have been found to have vitamin E activity, d-α, d-β, d-γ and d-δ tocopherol (Which differ in methylation site and side chain saturation) and d-α, d-β, d-γ and d-δ tocotrienol.

d-α tocopherol has the highest biopotency and its activity is the standard against which all others must be compared. It is the predominant isomer in plasma. Vitamin E is an essential nutrient that functions as an antioxidant in the human body. Absorption is believed to be associated with the intestinal fat absorption. Approximately 40% of the ingested tocopherol is absorbed.

The main function of tocopherol is to prevent the peroxidation of membrane phospholipids and avoid cell membrane damage through its antioxidant action.

α - tocopherol + LOO* ⟶ **α-tocophenol* + LOOH**

α-tocophenol + LOO ⟶ **LOO - α-tocophenol**

α-tocopherol has been shown to be capable of reducing ferric ion (i.e. to act as a pro-oxidant. Moreover, the ability of α-tocopherol to act as a pro-oxidant (reducing agent) or antioxidant depends on whether all of the α-tocopherol becomes consumed in the conversion from ferric to ferrous ion.

1.3.3.2. Ascorbic Acid

Ascorbic acid is a water soluble antioxidant present I citrus fruits, potatoes, tomatoes and green leafy vegetables. Humans are unable to synthesise L-ascorbic acid from d-glucose due to the absence of the enzyme L-galaco acetone oxidase. It is a water soluble chain breaking antioxidant. It scavenges free radicals and reactive oxygen molecules which are produced during metabolic pathways of detoxification. It also prevents formation of carcinogens from precursor compounds.

One important property is its ability to act as a reducing agent (Electron donor). Ascorbate is more potent than α-tocopherol in inhibiting the oxidation of low density lipoprotein (LDL) in a cell free system. The concentration of ascorbate use to inhibit LDL oxidation (40-60 μm) is within the normal plasma range. Vitamin C supplementation in animals leads to increased plasma and tissue levels of Vitamin E.

1.3.3.3. Coenzyme Q_{10}

It is an excellent antioxidant with characteristics similar to vitamin E. it is a powerful immune system stimulant. It is also known to have a great number of other useful characteristics including cardiovascular benefits, anti-ageing, gum disease and cellular energy.

1.3.4. Mechanism of action of Antioxidants

There are

- Physical barriers preventing ROS generation or ROS access to important biological sites. E.g. UV filters, cell membranes.
- Chemical traps/sinks 'absorb' energy and electrons quenching ROS. E.g. carotenoids, anthocyanidins.
- Catalytic system neutralize or diverts ROS. E.g. SOD, catalase and glutathione peroxidase.
- Binding/inactivation of metal ion prevents generation of ROS by Haber-Weiss reaction. E.g. Ferritin, caeruloplasmin, catechins.
- Sacrificial and chain breaking antioxidants scavenge and destroy ROS. E.g. Ascorbic acid (Vit. C), tocopherols (Vit. E), uric acid, glutathione and flavanoids.

2. 0 PHARMACOLOGICAL EVALUATION OF *DELONIX REGIA* (BOJ. EX HOOK.) RAF. LEAVES FOR ANTIMICROBIAL ACTIVITY

Medicinal plants are a source of great economic value in the Indian subcontinent. Herbal medicine is still the mainstay of about 75-80% of the whole population mainly in developing countries for primary health care because of better cultural acceptability, better compatibility with the human body and fewer side effects. However the last few years have seen a major increase in their use in the developed world.

Now a day multiple drug resistance has developed due to the indiscriminate use of commercial anti-microbial drugs commonly used in the treatment of infectious disease. Therefore there is a need to develop alternative anti-microbial drugs for the treatment of infectious disease from medicinal plants. In addition to this problem antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune suppression and allergic reaction.

Several screening studies have been carried out in different parts of the world. There are several reports on the anti-microbial activity of different herbal extracts in different regions of the world. This situation forced

scientist to search for new anti-microbial substances. Given the alarming incidence of antibiotic resistance in bacteria of medical importance, there is a constant need for new and effective therapeutic agents.

Because of the side effects and the resistance that the pathogenic microorganisms built against antibiotics, recently much attention has been paid to extracts and biologically active compounds isolated from plant species used in herbal medicine.

Anti-microbials of plant origin have enormous therapeutic potential. Plant-based anti-microbials represent a vast untapped source of medicines and further exploration of plant anti-microbials needs to occur. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic anti-microbials.

All plants containing active compounds are important. The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plants. In plants, these compounds are mostly secondary metabolites such as alkaloids, steroids, tannins and phenol compounds which are synthesized and deposited in specific parts or in all parts of plants. These compounds are more complex and specific and are found in certain taxa such as family, genus and species but the heterogeneity of secondary compounds is found in wild species.

In the present work the leaf ethanolic extract was evaluated for their anti-microbial properties using bacterias and fungi.

2.1. ANTIMICROBIAL EVALUATION OF *DELONIX REGIA* (BOJ. EX HOOK.) RAF. LEAVES [131&132]

The microbiological assay is based upon a comparison of the inhibition of growth of microorganisms by measured concentrations of the antibiotics to be examined with that produced by known concentrations of a standard preparation of the anti-biotic having a known activity.

The anti-microbial activity of *Delonix regia* (Boj. ex Hook.) Raf. leaf ethanolic extracts were studied by the presence of zones of inhibition against microorganisms.

The following microorganisms were used for our studies.

- **Strepto cocci**
- *Staphylo cocci*
- *Proteus vulgaris*
- *Escherichia coli*
- *Pseudomonas aeuroginosa*
- *Klebsiella aerugenes*
- *Candida albicans*

5. PREPARATION OF NUTRIENT BROTH FOR BACTERIA

Ingredients:

Beef extract	1gm
Peptone	1 gm
Sodium chloride	0.5 gm
Distilled water	100 ml

Procedure:

The accurately weighed quantities of above ingredients were dissolved in distilled water and pH was adjusted to 7.4 and sterilized by autoclaving.

PREPARATION OF SABOURAUD DEXTROSE BROTH FOR FUNGI

Ingredients:

Dextrose	-	4gm
Peptone	-	1gm
Distilled Water	-	100ml

Procedure:

The weighed quantities of ingredients were dissolved in distilled water and pH was adjusted to 5.2 and sterilized by autoclaving.

PREPARATION OF SLANTS

To the broth 2% of agar was added to prepare nutrient agar and sabouraud dextrose agar media.

PREPARATION AND STANDARDIZATION OF INOCULUM

Each bacterial and fungi pure cultures were transferred into 100ml of nutrient broth (NB) and Sarbouraud's Dextrose Broth (SDB) respectively. The inoculated broths were incubated at 37℃ fro 24 hours and 27℃ for 72 hours fro bacterial and fungi respectively. After incubation inoculum were standardized to 10^8 CFU/ml for bacteria and 10^6CFU/ml for fungi by colony forming unit method.

2.2 ANTIMICROBIAL ACTIVITY OF *DELONIX REGIA* (BOJ. EX HOOK.) RAF. BY DISC DIFFUSION METHOD

Sample Preparation

The ethanolic extract of *DELONIX REGIA* (BOJ. EX HOOK.) RAF**.** was dissolved in 10% Dimethyl formamide (DMF) to a final concentration of 50mg/ml, 75mg/ml, 100mg/ml and 200mg/ml. The sterile discs (6mm in diameter) were impregnated with 10µl of the extracts and tested against

microbial cultures. (100 sterile discs were taken and soaked in 1ml of extracts of various concentrations).

Antibacterial Activity

Muller Hinton agar medium was prepared and transferred into sterile petriplates. 200µl of the standardized bacterial inoculum was spread on agar medium using sterile cotton swab. The extracts impregnated discs were placed on the inoculated agar medium. Negative control was prepared using the same solvent employed to dissolve the plant extracts. Amikacin 5µg/disc was used as positive reference standard to determine the sensitivity of each microbial species tested. All the petriplates were incubated at 37^0C for 24 hours. After the incubation diameter of zone of inhibition was measured.

Anti fungal Activity

Sarbouraud's dextrose agar medium was prepared and transferred into sterile petriplates. 200µl of the standardized fungal inoculum was spread on agar medium using sterile cotton swab. The extracts impregnated discs were placed on the inoculated agar medium. Negative control was prepared using the same solvent employed to dissolve the plant extracts. Ketoconazole 10µg/disc was used as positive reference standard to determine the sensitivity of each microbial species tested. All the petriplates were

incubated at 27^0C for 72 hours. After the incubation diameter of zone of inhibition was measured.

Table: 28 Anti- microbial activities of D*elonix regia* (Boj. Ex Hook.) Raf. by disc diffussion method

S.NO	*MICROORGANISMS*	*ZONE OF INHIBITION* (ZI) in mm				
		500µg	**750µg**	**1000µg**	**2000µg**	**Std**
I	***BACTERIA***					***Amikacin 5µg/disc***
01	**Strepto cocci**	--	--	12	14	**16**
02	***Staphylococcus aureus***	--	10	11	13	**15**
03	***Proteus vulgaris***	--	--	--	--	**18**
04	***Escherichia coli***	--	10	12	14	**16**
05	***Pseudomonas aeuroginosa***	18	20	24	26	**22**
06	***Klebsiella aerugenes***	--	--	10	11	**22**
II	***FUNGI***					***Ketoconazole 10µg/disc***
01	***Candida albicans***	--	--	--	17	**16**

-- No inhibition zone

3.0 PHARMACOLOGICAL EVALUATION OF ISOLATED COMPOUNDS S_8 AND F_1 FOR *IN VITRO* AND *EX VIVO* ANTIOXIDANT ACTIVITIES

The isolated Sterol S_1 and the flavanoid F_1 were subjected for their antioxidant potential by means of *in vitro and ex vivo* antioxidant studies. The various methods employed were as follows:

***In Vitro* Methods**

- ❖ Reducing power ability.
- ❖ Nitric Oxide radical scavenging assay.
- ❖ DPPH radical scavenging assay

Ex Vivo Methods

- ➢ Assay of Lipid Peroxidation method.

Before determining the antioxidant potential of the isolated compounds, the Total Phenolics content of the crude alcoholic extract was determined in order to predetermine the antioxidant potential of the plant or not.

3.1. Estimation of Total Phenolic Content of *Delonix regia (Boj. ex Hook.) Raf.* leaves[117&118]

Total soluble Phenolics of the extracts were determined with Folin-Ciocalteu reagent using gallic acid as a standard. 100mg of the extract was extracted with 20ml of methanol (20*5) and filtered. The filtrate is made up to 100ml with methanol. 2 ml of the content is taken and made up to 3ml by addition 1 ml of water. To the contents add 0.5ml of Folin-Ciocalteu reagent and kept aside for 3 min. to this add 2ml of 20% sodium carbonate solution and mixed thoroughly. The contents were placed in boiling water bath for 1 min and cooled down. In the similar way the standard gallic acid is also prepared using 50mg of gallic acid. The absorbance of standard and sample were measured at 650nm against blank.

$$\text{Total Phenolics} = \frac{\text{Sample reading X Standard weight X Sample dilution x 100}}{\text{Standard reading X Standard dilution X Sample weight}}$$

$$\text{Total Phenolic content} = \frac{1.5423 \text{ X } 52.3 \text{ X } 5 \text{ X } 100 \text{ X } 100}{0.7954 \text{ X } 50 \text{ X } 50 \text{ X } 102}$$

Total Phenolic content= 19.8845mg/100gms as Gallic acid equivalents.

3.2. Antioxidant Methods

3.2.1. In Vitro Methods

3.2.1.1. Reducing power ability[119-121]

The reducing power ability of S_8 and F_1 was investigated by the Fe3+ - Fe2+ transformation in the presence of extract. The reductive ability was measured by mixing 1.0 ml of the sample prepared with distilled water to 2.5 ml of phosphate buffer (0.2 m, pH 6.6) and 2.5 ml of 1% potassium ferricyanide and incubated at 50℃ for 30 min. After that 2.5 ml of trichloroacetic acid (10%) were added to the mixture and centrifuged for 10 min at 3000g, 2.5 ml from the upper part were diluted with 2.5ml of water shaken with 0.5ml of fresh 0.1% ferric chloride. The absorbance was measured at 700nm using UV spectrophotometer. The reference solution was prepared as above and contain water instead of the samples. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C is used as the standard.

Table: 29 **Total Reducing ability of S_8 and F_1 of *Delonix regia* (Boj. ex Hook.) Raf leaves**

6. **Control = 0.021**

7. S	Sample	Concentration µg/ml	Absorbance
01	S_8	125	0.046 ± 0.0023*
		250	0.068 ± 0.0017***
		500	0.076 ± 0.0008***
		750	0.086 ± 0.0020***
		1000	0.100 ± 0.001***
02	F_1	125	0.064 ± 0.0067***
		250	0.099 ± 0.0029***
		500	0.109 ± 0.0015***
		750	0.124 ± 0.0046***
		1000	0.145 ± 0.004***
03	Vit C	01	0.076 ± 0.0054***
		02	0.1117 ± 0.0015***
		03	0.1563 ± 0.0075***
		04	0.1803 ± 0.005***
		05	0.2270 ± 0.007***

Values are Mean ± SEM n = 03

*** P < 0.001 as compared to the Control group

* P < 0.05 as compared to the control group

3.3.1.2. DPPH radical scavenging assay[120,122-125]

The free radical scavenging activity of isolated compounds S_8 and F_1 of *Delonix regia (Boj. ex Hook.) Raf.* was measured in vitro by 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay. The hydrogen donating ability of the compounds were determined in the presence of DPPH stable radical. The isolated compounds and stock solution (1mg/ml) was diluted to required concentrations in methanol. One milliliter of 0.1mM DPPH methanol solution was added to 1.0ml of sample solution of different concentration and allowed to react at room temperature. After 30min the absorbance was measured at 517 nm. The values obtained were converted into percentage antioxidant activity (AA%) using the following formula

$$AA\% = \left(\frac{[(Abs_{Control} - Abs_{Sample}) \times 100]}{Abs_{Control}} \right)$$

Vit. C (Ascorbic acid) is used as the standard. The IC_{50} values are calculated by linear regression of plots.

Table: 30 DPPH radical scavenging assay of S_8 and F_1 of *Delonix regia* (Boj. ex Hook.) Raf leaves

8. **Control = 0.4810**

9. S	Sample	Concentration (µg/ml)	% Inhibition	10. IC_{50}
01	S_8	125 250 500 750 1000	20.09 ± 2.069 31.11 ± 2.285 41.79 ± 1.590 50.94 ± 2.542 63.54 ± 1.434	720
02	F_1	125 250 500 750 1000	30.55 ± 2.911 46.42 ± 1.988 65.62 ± 2.609 78.31 ± 0.8133 87.31 ± 1.477	300
03	Vit C	01 02 03 04 05	26.26 ± 1.330 39.08 ± 0.9836 48.16 ± 4.429 65.90 ± 2.359 80.52 ± 0.6838	3.1

Values are Mean ± SEM n = 03

P < 0.001 as compared to the Control group

3.3.1.3. **Nitric oxide radical scavenging assay**[120, 126&127]

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitric ions, which were measured by Griess reagent. The reaction mixture with a final volume of 3 ml per tube containing 2.0 ml of 10 mM sodium nitro prusside in phosphate buffer saline solution and was incubated with 1.0 ml of different concentration of test compounds (S_8 and F_1) dissolved in phosphate buffer (0.025 M, pH 7.4) and the tubes were incubated at 25℃ for 150min. Control experiments were carried out without the test compounds but with equivalent amount of buffer solution. There after 0.5ml of incubation solution was removed and diluted with 0.5ml of Griess reagent (1% Sulphanilamide in 2% O –Phosphoric acid and 0.1% napthylene diamine hydrochloride) and allowed to react for 30 min. The absorbance of the chromophore formed during diazotisation of nitrite with sulphanilamide and subsequent coupling with napthylene diamine dihydrochloride was read at 546nm. The percentage inhibition was calculated using the formula

$$\text{Percentage inhibition (I)} = \frac{(A_o - A_1)}{A_o} \times 100$$

Where A_o is the absorbance of the control reaction and A_1 is the absorbance of the test compound. The experiment was done using Vitamin C as Standard.

Table: 31 **Nitric Oxide radical scavenging assay of S_8 and F_1 of *Delonix regia* (Boj. ex Hook.) Raf leaves**

11.Control = 0.4476

S.No	Sample	Concentration (µg/ml)	% Inhibition	IC_{50}
01	S_8	125 250 500 750 1000	1.197 ± 0.3382 5.093 ± 1.524* 47.86 ± 3.028*** 63.91 ± 1.444*** 67.30 ± 1.961***	530
02	F_1	125 250 500 750 1000	29.75 ± 1.5970*** 46.83 ± 1.135*** 64.50 ± 0.4421*** 76.37 ± 0.8580*** 80.82 ± 0.8228***	310
03	Vit C	01 02 03 04 05	20.73 ± 1.7840*** 34.52 ± 0.8792*** 56.42 ± 0.9635*** 73.04 ± 0.1637*** 78.22 ± 0.5685***	2.7

Values are Mean ± SEM n = 03

*** P < 0.001 as compared to the Control group

* P < 0.05 as compared to the control group

3.3.2. Ex Vivo Methods

3.3.2.1. Estimation of TBAR's (Lipid Peroxidation Method)[128-130]

TBA reacts with malonyldialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532nm. The liver homogenate were prepared from normal male rats (250gms). The perfused liver was isolated and 10% w/v homogenate was prepared with homogenizer with 0.15M KCl. The homogenate was centrifuged at 800g for 15min and clear cell free supernatant was used for the study of in vitro lipid peroxidation. Various concentrations of the isolated compounds of S_8 and F_1 are dissolved in methanol and taken in test tubes. One ml of 0.15M KCl and 0.1ml of homogenate were added to the test tubes. Peroxidation was initiated by adding 100µl of 0.2mM ferric chloride. After incubation at 37℃ for 30min, the reaction was stopped by adding 2ml of ice cold hydrochloric acid (0.25N) containing 15% trichloroacetic acid (TCA), 0.38% TBA and 0.5% BHT. The reaction mixture was heated at 80℃ for 60min. The samples were cooled and centrifuged and the absorbance of the supernatant was measured at 532nm.

$$\text{Percentage inhibition (I)} = \frac{(A_o - A_1)}{A_o} \times 100$$

Where A_o is the absorbance of the control reaction and A_1 is the absorbance of the test compound. The experiment was done using Vitamin C as Standard.

Table: 32 Lipid peroxidation assays of S_8 and F_1 of *Delonix regia* (Boj. ex Hook.) Raf leaves

12.Control = 0.4218

S.No	Sample	Concentration (µg/ml)	% Inhibition	IC_{50}
01	S_8	125	03.44 ± 1.116	520
		250	14.50 ± 2.356***	
		500	47.91 ± 0.44***	
		750	62.97 ± 1.750***	
		1000	75.53 ± 1.396***	
02	F_1	125	19.21 ± 3.573**	480
		250	40.33 ± 1.376***	
		500	50.99 ± 0.8967***	
		750	65.88 ± 1.644***	
		1000	84.25 ± 1.096***	
03	Vit C	25	32.63 ± 2.330***	60
		50	47.23 ± 0.6253***	
		100	61.75 ± 1.397***	
		250	70.82 ± 0.7988***	
		500	87.12 ± 1.819***	

Values are Mean ± SEM n = 03

*** P < 0.001 as compared to the Control group

- P < 0.05 as compared to the control group.
-

PHYTOCHEMICAL INVESTIGATION

01. COLLECTION AND AUTHENTICATION

Delonix regia (Boj. ex Hook.) Raf. leaves were collected from in and around areas of Madurai Medical College campus, Madurai and the same was authenticated by **Dr. D. Stephen, M. Sc, Ph.D,** Professor & HOD, Department of Botany, The American college, Madurai.

02. MACRO-MORPHOLOGICAL STUDY[76]

Morphology is the study of the form of an object whilst morphography is the description of that form. A number of different bases may be used on the classification of drugs and morphological studies play a great role in this respect. The application of morphology in drug analysis lies in the field of crude drugs where the material is known to occur in a particular form, it can be studied for the whole drug i.e., macro-morphography or gross morphology study of cell characteristics i.e. cytomorphology or study of morphological character in a particular level.

The majority of natural products used as drugs are derived from plants or parts of plants. Characteristically the higher plants consists in the vegetative phase of roots, stems, leaves with flowers, fruits and seeds in the reproductive cycle. Modifications of the above structures are frequently present such as rhizomes (underground stems), stipules, bracts (modified leaves), tendrils (modified stem), stolons
(runners with a stem structure) etc., Interpretations of the morphological characteristics based on different parameters, for all the plant organs as above gives us a first hand tool to know the features of whole or powdered drugs and adulterants of commercial significance.

It is most useful only for only a part of plant to be used, either because the active constituents is only found in a particular part or because of economic consideration, which dictate the collection of certain parts of the whole plant. Most species of a plant are readily distinguishable in their natural state, but collection, preparation and drying procedure distortion of that natural form, making recognition more difficult. Natural variations in size and shape are common because of the environmental factors where

Macro-morphology plays an important role for preliminary evaluation of the crude drugs. Based upon the anatomical structure of the plant the groups or the parts of plant commonly used for different therapeutic categories are leaves, bark, flowers, fruits and seeds, wood, herb or aerial parts, sub terranean organ etc.

Table: 01 Macro-morphological evaluation of *Delonix regia* (Boj. ex Hook.) Raf leaf

S.No	PARAMETER	MORPHOLOGY
Leaf - Compound		
01	Surface	Glabrous
02	Lamina	Oval
03	Margin	Entire
04	Apex	Obtuse
05	Base	Slightly asymmetrical
06	Venation	Reticulate

03. ORGANOLEPTIC EVALUATION OF POWDERED LEAF

Colour : Dark green.

Odour : Odourless.

Taste : Slightly bitter.

Texture : Gritty.

Size : Uneven sized coarse particles.

04. PHYSICAL CONSTANTS [77&78]

The following physical constants were carried out for the leaf and flower powder:

1. n – Hexane soluble extractive (HSE)
2. Petroleum ether soluble extractive 40 - 60 (PESE).
3. Chloroform soluble extractive (CSE).
4. Ethyl acetate soluble extractive (EASE).
5. Acetone soluble extractive (ASE).
06. Methanol soluble extractive (MSE).
07. Water soluble extractive (WSE).
8. Total ash
9. Sulphated ash.

4. 1. EXTRACTIVE VALUES

This method determines the amount of active constituents in a given amount of medicinal plant material when extracted with solvents. It is employed for that material for which no chemical or biological assay method exists. As mentioned in different official books the determination of water-soluble and alcohol soluble extractives, is used as a means of evaluating crude drugs which are not readily estimated by other means.

The extraction of any drug with a particular solvent yields a solution containing different phytoconstituents. The composition of these phytoconstituents in that particular solvent depends upon the nature of the drug and solvent used. The use of a single solvent can be the means of providing preliminary information on the quality of a particular drug sample; for example, in a drug where the extraction procedure for the constituents commences with water as the solvent, any subsequent aqueous extraction on the re-dried residue will give a very low yield of soluble matter.

4.1.1 n-Hexane soluble extractive [HSE]

5gms of the air dried powder of *Delonix regia* was macerated with 100ml of n – Hexane in a stoppered flask, shaking frequently during the first 6 hours and allowed to stand for 18 hrs. Thereafter it was filtered rapidly taking precaution against the loss of solvent. Evaporate 25 ml of the filtrate to dryness in a tarred flat bottom shallow dish dried at 105°C, and weighed. Percentage n-Hexane soluble extractive was calculated with reference to the air-dried plant.

4.1.2 Petroleum ether soluble extractive [PESE].

5gms of the air dried powder of *Delonix regia* was macerated with 100ml of Petroleum ether 40-60 in a stoppered flask, shaking frequently during the first 6 hours and allowed to stand for 18 hrs. Thereafter it was filtered rapidly taking precaution against the loss of solvent. Evaporate 25 ml of the filtrate to dryness in a tarred flat bottom shallow dish dried at 105°C, and weighed. Percentage Petroleum ether soluble extractive was calculated with reference to the air-dried plant.

4.1.3 Chloroform Soluble Extractive [CSE]

5gms of the air-dried powder of *Delonix regia* was macerated with 100ml of chloroform in a stoppered flask, shaking frequently during the first 6 hours and allowed to stand for 18 hrs. Thereafter it was filtered rapidly taking precaution against the loss of solvent. Evaporate 25 ml of the filtrate to dryness in a tarred flat bottom shallow dish dried at 105^{o}C and weighed. Percentage n-Hexane soluble extractive was calculated with reference to the air-dried plant.

4.1.4 Ethyl Acetae Soluble extractive [EASE].

5gms of the air-dried powder of *Delonix regia* was macerated with 100ml of ethyl acetate in a stoppered flask, shaking frequently during the first 6 hours and allowed to stand for 18 hrs. Thereafter it was filtered rapidly taking precaution against the loss of solvent. Evaporate 25 ml of the filtrate to dryness in a tarred flat bottom shallow dish dried at 105^{o}C, and weighed. Percentage ethyl acetate soluble extractive was calculated with reference to the air-dried plant.

4.1.5 Acetone soluble extract [ASE]

5gms of the air-dried powder of *Delonix regia* was macerated with 100ml of acetone in a stoppered flask, shaking frequently during the first 6 hours and allowed to stand for 18 hrs. Thereafter it was filtered rapidly taking precaution against the loss of solvent. Evaporate 25 ml of the filtrate to dryness in a tarred flat bottom shallow dish dried at 105°C, and weighed. Percentage acetone soluble extractive was calculated with reference to the air-dried plant.

4.1.6 Methanol Soluble Extractive. [MSE]

5gms of the air-dried powder of *Delonix regia* was macerated with 100ml of methanol in a stoppered flask, shaking frequently during the first 6 hours and allowed to stand for 18 hrs. Thereafter it was filtered rapidly taking precaution against the loss of solvent.

Evaporate 25 ml of the filtrate to dryness in a tarred flat bottom shallow dish dried at 105°C, and weighed. Percentage methanol-soluble extractive was calculated with reference to the air-dried plant.

4.1.7 Water Soluble Extractive. [WSE]

5gms of the dried powder of *Delonix regia* was added into 50ml of boiled water at 80°C in a stoppered flask. It was shaken well and allowed to cool, filtered. 5ml of filtrate was transferred to a tarred evaporating dish, the solvent was evaporated on a water bath, allow to dry for 30min. Finally dried in an oven for 2 hrs at 100°C and residue was weighed. Percentage of water-soluble extractives was calculated with reference to the air-dried crude drug.

Calculations[79]:

25 ml of extract gives = X gm of residue.

For 100ml of extract gives = 4 * X of residue.

5 gm of air dried drug gives 4 * X gm of residue,

Then 100gms of air-dried drug gives 20 * 4 * X gm of residue.

Therefore 100gms of air-dried drug gives 80 X %

Table:02 Extractive values of D*elonix regia* (Boj. ex. Hook.) Raf leaf

S.No	Extractives	Determined extractive values (% w/w)
01	n-Hexane	02.938
02	Petroleum ether 40-60	04.935
03	Chloroform	03.732
04	Ethyl acetate	07.865
05	Acetone	05.681
06	Methanol	21.249
07	Water	47.80

4. 2. ASH VALUES

The ash of any material is composed of their non-volatile inorganic components. Controlled incineration of crude drugs results in an ash residue consisting of an inorganic material (metallic salts and silica). This value varies within fairly wide limits and is therefore an important parameter for the purpose of evaluation of crude drugs. In certain drugs, the percentage variation of the weight of ash from sample to sample is very small and any marked difference indicates a change in quality. Unwanted parts of drugs, sometimes, possess a character that will raise the ash value-for example, the sclerides in the unwanted pericarp of colocynth and the cork on liquorice, which is not required in the powder of the peeled drug. More direct contamination, such as by sand or earth, is immediately detected by the ash value. The ash value can be determined by three different methods to measure the total ash, the acid insoluble ash and the water soluble ash.

4.2.1 Total Ash

Weigh accurately a quantity of the powdered drug equivalent to 1 gm of the air dried drug in a tarred platinum silica dish and incinerate a temperature not exceeding 550°C until free from carbon, approximately for about 4hrs, cool and weigh. Calculate the percentage of ash with reference to the air-dried drug.

4.2.2 Sulphated Ash

Heat a silica or platinum crucible to redness for 10minutes and allow cooling in a desicator and weigh. Put 1gm of the substance accurately weighed into crucible and ignite gently at first. Cool moisten the residue with 0.5ml of Sulphuric acid, heat at 550℃ for about 4 hrs. Cool and weigh, calculate the percentage of ash with reference to the air-dried drug.

Table : 03 **Ash values of D*elonix regia* (Boj. ex Hook.) Raf. leaves**

S.No	Ash	Percentage ash value (w/w)
01	Total ash	07.40
02	Sulphated ash	07.19

05. PHYTO CHEMICAL INVESTIGATION

A. Method of extraction

Fresh leaf of *Delonix regia* were collected shade dried at room temperature, pulverized and extracted with 95% ethanol in a soxhlet extractor. The extract was concentrated in a rotary flash evaporator. The residue was dried in a desicator over sodium sulphite

Table: 04

Percentage extractives & physical characteristics of alcoholic extract of D*elonix regia* (Boj. ex Hook.) Raf.

S.NO	EXTRACT	% DRY WEIGHT	COLOUR	ODOUR	CONSISTENCY
01	Crude alcoholic	25.85	Dark green	Characteristic	Sticky

B. QUALITATIVE PHYTOCHEMICAL ANALYSIS [76, 79 –85]:

The qualitative chemical tests for various phytoconstituents were carried out using the leaf alcoholic extract of *Delonix regia*

5. B. 1. Test for ALKALOIDS

i. **Mayer's test :** Alkaloids give cream colour precipitate with Mayer's Reagent [Potassium mercuric iodide solution].

ii. **Dragondroff's test :** Alkaloids give reddish brown precipitate with Dragondroff's reagent [Potassium bismuth iodide solution].

iii. **Wagner's test :** Alkaloids give a reddish brown precipitate with Wagner's reagent. [Solution of iodine in potassium iodide].

iv. **Hager's test :** Alkaloids give yellow colour precipitate with Hager's reagent[Saturated solution of Picric acid].

v. **Tannic acid test :**Alkaloids give buff colour precipitate with 10% Tannic acid solution.

5. B. 2. Test for GLYCOSIDES :

General test for the presence of Glycosides:

Test A : Extract 200mg of the drug by warming in a test tube with 5ml of dilute(10%) sulfuric acid on a water bath at 100°C for 2min, centrifuge or filter, pipette off supernatant or filtrate. Neutralize the acid extract with 5% solution of Sodium hydroxide (noting the volume of NaOH added). Add 0.1ml of Feghling's solution A and then B until alkaline(test with pH paper) and heat on a water bath for 2min. Note the quantity of red precipitate formed and compare with that formed in Test B.

Test B: Extract 200mg of the drug using 5ml of water instead of sulfuric acid. After boiling add volume of water equal to the volume of sodium hydroxide used in the above test. Add 0.1ml of Feghling's solution A and B until alkaline (test with pH paper) and heat on water bath for 2min. Note the quantity of red precipitate formed.

Compare the quantity of precipitate formed in Test B with that of formed in Test A. If the precipitate in Test A is greater than in Test B then Glycoside may be present. Since Test B represents the amount of free reducing sugar already present in the crude drug, Whereas Test A represents free reducing sugar plus those related on acid hydrolysis of any Glycosides in the crude drug.

5. B. 2.1. General tests for free sugars:

After complete removal of free sugars, the extract is hydrolyzed with mineral acid

and then tested for the glycone and aglycone moieties.

a. **Raymond's test:** Test solution when treated with dinitrobenzene in hot methanolic alkalie, gives violet color.

b. Legal's test: Treat the extract with pyridine and add alkaline sodium nitroprusside solution, blood red color appears.

c. Bromine water test: Test solution when treated with bromine water gives yellow precipitate.

5. B. 2. 2. Chemical tests for specific glycosides:

5. B. 2. 2. A. Test for SAPONIN GLYCOSIDES:

Froth Test: Place 1ml solution of drug in water in a semi-micro tube shake well and note the stable froth.

Haemolysis test: Add 0.2ml solution of saponin(prepared in 1% normal saline) to 0.2ml of V/V blood in normal saline and mix well, centrifuge and note the red supernatant compare with control tube containing 0.2ml of 10% blood in normal saline diluted with 0.2ml of normal saline.

5. B. 2. 2. B. Test for ANTHRAQUINONE GLYCOSIDES:

Borntragers test: Boil test material with 1ml of dil.sulphuric acid in a test tube for 5min (anthracene glycosides are hydrolyzed to aglycone and sugars by boiling with acids) centrifuge or filter while hot (if centrifuged hot, the plant material can be removed while anthracene aglycone are still sufficiently soluble in hot water, they are however insoluble in cold water), pipette out the supernatant or filterate, cool and shake with an equal volume of dichloromethane (the aglycone will dissolve preferably in

dichloromethane) separate the lower dichloromethane layer and shake with half its volume with dilute ammonia. A rose pink to red color is produced in the ammoniacal layer (aglycones based on anthroquinones give red color in the presence of alkali).

Modified Borntragers test: Boil 200mg of the test material with 2ml of dilute sulphuric acid, 2ml of 5% aqueous ferric chloride solution for 5min and continue the test as above. As some plant contains anthracene aglycone in a reduced form, if ferric chloride is used during the extraction, oxidation to anthroquinones takes place, which shows response to the Borntrager's test.

5. B. 2. 2. C. Test for CARDIAC GLYCOSIDES:

Kedde's test: Extract the drug with chloroform, evaporate to dryness, add one drop of 90% alcohol and 2 drops of 2% 3,5-dinitro benzoic acid(3,5-dinitro benzene carboxylic acid-Kedde's reagent) in 90% alcohol. Make alkaline with 20% sodium hydroxide solution. A purple color is produced. The color reaction with 3,5- dinitrobenzoic acid depends upon the presence of an α,β -unsaturated-δ lactones in the aglycone.

Keller killiani test[test for Deoxy sugars]: Extract the drug with chloroform and evaporate it to dryness. Add 0.4ml of glacial acetic acid containing a trace amount of ferric chloride. Transfer to a small test tube; add carefully 0.5ml of concentrated sulphuric acid by the side of the test tube, blue color appears in the acetic acid layer.

5. B. 2. 2. D. Test for CYANOGENETIC GLYCOSIDES:

Place 200mg of drug in a conical flask and moisten with few drops of water, there should be no free liquid at the bottom of the flask (the test will not work if there is any liquid in the flask as the hydrogen cyanide produced will dissolve in the water rather than, come off as a gas to react with the paper). Moisten a piece of picric acid paper with sodium carbonate solution (5% aqueous) and suspended by means of cork in the neck of the flask, warm gently at about 37^{o}C. Observe the change in color. Hydrogen cyanide is liberated from cyanogenetic glycoside by the enzyme activity and reacts with sodium picrate to form the reddish purple sodium isopicrate.

5.B.3. Test for PHENOLIC COMPOUNDS (TANNINS, FLAVONOIDS & OTHER PHENOLS)

Due to presence of vicinyl oxygenated function many of the compounds acts as legend for metal ions. Thus ferric ions when added to solution of phenols produce dark green, blue or blue-black complex.

5. **B. 3. 1. <u>Test for TANNINS:</u>**

01. Gelatin test: Test solution with 1% gelatin solution containing 10% sodium chloride gives white precipitate.

02. Ferric chloride test: Test solution give blue green color with Ferric chloride.

03. Vanillin Hydrochloride test: Test solution when treated with few drops of Vanillin Hydrochloride reagent gives purplish red color.

04. Tannins get precipitated in the solution when treated with heavy metals.

05. Tannins yield bulky precipitate with phenazone specially in the presence of sodium and phosphate.

06. Alkaline reagent test: Test solution with sodium hydroxide solution gives yellow to red precipitate within short time.

07. Metchell's test: With iron and ammonium citrate or iron and Sodium tartarate Tannins give a water-soluble iron-tannin complex which is insoluble in solution of Ammonium acetate.

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5. B. 3. 2. Test for FLAVONOIDS:

Shinoda test (Magnesium Hydrochloride reduction test): To the test solution add few fragments of Magnesium ribbon and add conc. Hydrochloric acid drop wise, pink scarlet, crimson red or occasionally green to blue color appears after few minutes.

Zinc Hydrochloride reduction test: To the test solution add a mixture of Zinc dust and conc. Hydrochloric acid. It gives red color after few minutes.

Alkaline reagent test: To the test solution add few drops of sodium hydroxide solution; formation of an intense yellow color which turns to colorless on addition of few drops of dil.acid indicates presence of Flavonoids.

5. B. 4. Test for PROTEINS & AMINOACIDS:

Millons test: Test solution with 2ml of Millons reagent (Mercuric nitrate in nitric acid containing traces of nitrous acid), white precipitate appears, which turns red upon gentle heating.

Ninhydrin test: Amino acids and Proteins when boiled with 0.2% solution of Ninhydrin (Indane 1,2,3 trione hydrate), Violet color appears.

5. B. 5. Test for STEROLS & TRITERPENOIDS:

Libermann-Buchard test: Extract treated with few drops of acetic anhydride, boil and cool, conc. Sulfuric acid is added from the sides of the test tube, shows a brown ring at the junction of two layers and the upper layer turns green which shows the presence of Steroids and formation of deep red color indicates the presence of triterpenoids.

Salkowski test: Treat extract in Chloroform with few drops of conc. Sulfuric acid, shake well and allow to stand for some time, red color appears at the lower layer indicates the presence of Steroids and formation of yellow colored lower layer indicates the presence of Triterpenoids.

5. B. 6. Test for CARBOHYDRATES:

Molisch's test: Treat the test solution with few drops of alcoholic alpha napthol. Add 0.2ml of conc. Sulfuric acid slowly through the sides of the test tube, a purple to violet color ring appears at the junction.

Benedict's test: Treat the test solution with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) and upon boiling on water bath, reddish brown precipitate forms if reducing sugars are present.

Barfoed's test: It is a general test for Mono-saccharides. Heat the test tube containing 1ml of reagent and 1ml of solution of compound in a beaker of boiling water; if red cuprous oxide is formed within 2min, mono-saccharides is present. Disaccharide on prolonged heating(about 10min) may also cause reduction, owing to partial hydrolysis to mono-saccharides.

Caramelisation: Carbohydrates when treated with strong sulfuric acid, they undergo charring with the dehydration along with burning sugar smell.

Selwinoff's test: Hydrochloric acid reacts with ketose sugar to form derivatives of furfuraldehyde, which gives red colored compound when linked with resorcinol. Add compound solution to about 5ml of reagent and boil. Fructose give red color within half minute. The test is sensitive to 5.5mmol/ltr if glucose is absent, but if glucose is present it is less sensitive and in addition of large amount of glucose can give similar color.

Tollen's test: To 100mg of compound add 2ml of Tollen's reagent and heat gently, a silver mirror is obtained inside the wall of the test tube, indicates the presence of aldose sugar.

Bromine water test: It gets decolorized by aldose but not by ketoses because bromine water oxidizes selectively the aldehyde group to carboxylic group, giving raise to general class of compounds called aldonic acid.

Fehling's test: Equal volume of Fehling's A (Copper sulfate in distilled water) and Fehling's B (Potassium tartarate and Sodium hydroxide in distilled water) reagents are mixed and few drops of sample is added and boiled, a brick red precipitate of cuprous oxide forms, if reducing sugars are present.

5. B. 7. Test for FATS & FIXED OILS:

Stain test: Press the small quantity of extract between two filter papers, the stain on filter paper indicates the presence of fixed oils.

Saponification test: Add a few drops of 0.5N of alcoholic potassium hydroxide to small quantities of various extracts along with a drop of Phenolphthalein separately and heat on a water bath for 1-2hrs. The formation of soap or partial neutralization of alkali indicates the presence of Fixed oils and Fats.

5. B. 8. Test for VIT. C:

Sodium nitroprusside test: When test solution is treated with sodium - nitroprusside solution, a blue color is produced.

Sodium bicarbonate test: When test solution is treated with sodium - bicarbonte solution, a violet color is produced.

Table: 05 **Qualitative chemical examination of leaf alcoholic extract of D*elonix regia* (Boj. ex Hook.) Raf.**

S.No	PHYTOCONSTITUENTS	ALCOHOLIC EXTRACT
01	**ALKALOIDS**	**Present**
02	**FLAVONOIDS**	**Present**
03	**GLYCOSIDES**	**Present**
04	**CARBOHYDRATES**	**Present**
05	**PROTEINS &AMINO ACIDS**	**Present**
06	**VIT.C**	**Nil**
07	**TANNINS**	**Present**
08	**STEROLS**	**Present**
09	**TRITERPENOIDS**	**Present**
10	**SAPONINS**	**Nil**

06. TLC PROFILES FOR VARIOUS PHYTOCONSTITUENTS:

06. 1. TLC Profile Of Alkaloids[86]

Stationary phase : Silica Gel GF254

Mobile phase : Toluene (7): Ethyl acetate (2) : Diethyl amine (1)

Sample : Alcoholic extract.

Solvent front : 14.5 cm.

Detecting agent : UV detector for TLC at 365nm.

Table: 06 ***TLC Profile of Alkaloids***

Spot No	Visible Detection (Colour of the Spot)	UV_{365} Detection (Colour of the spot)	Distance traveled (Cm)	R_f
01	---	Pale green	1.5	0.10
02	---	Pale yellow	1.9	0.13
03	Light Yellow	Dark Blue	5.0	0.35
04	Dark Yellow	Dark Blue	6.2	0.43
05	Dark Green	Pink	8.0	0.55
06	Light Green	Pink	8.6	0.59

06. 2. TLC Profile Of Flavanoids[86]

Stationary phase : Silica Gel GF254

Mobile phase : Toluene (8) : Ethyl acetate (2)

Sample : Alcoholic extract.

Solvent front : 14.0 Cm

Detecting agent : UV detector for TLC at 365nm.

Table: 07 ***TLC Profile of Flavanoids***

Spot No	Visible Detection (Colour of the Spot)	UV_{365} Detection (Colour of the spot)	Distance traveled (Cm)	R_f
01	---	Light Rose	8.2	0.59
02	Light Yellow	Pink	9.0	0.64
03	Green	Dark Rose	9.2	0.66
04	Green	Dark Rose	9.4	0.67
05	Light Yellow	Pink	9.6	0.69
06	Grey	Pink	10.0	0.71
07	Light Yellow	Rose	11.0	0.79
08	Green	Dark Pink	11.2	0.80

06. 3. TLC Profile Of Glycosides[86]

Stationary phase : Silica Gel GF254

Mobile phase : Ethyl acetate (81) : Ethanol (11) : Water (08)

Sample : Alcoholic extract.

Solvent front : 15.0 cm.

Detecting agent : UV detector for TLC at 365nm.

Table: 08 ***TLC Profile of Glycosides***

Spot No	Visible Detection (Colour of the Spot)	UV_{365} Detection (Colour of the spot)	Distance traveled (cm)	R_f
01	---	Violet	1.0	0.07
02	---	Violet	6.0	0.40
03	Dark Brown	Pale yellow	6.3	0.42
04	---	Violet	10.0	0.67
05	Light Brown	Pale green	11.0	0.73
06	Light Yellow	Brown	12.2	0.81
07	Green	Pale yellow	13.8	0.92
08	---	Pink	14.8	0.98

06. 4. TLC Profile Of Sterols[87]

Stationary phase : Silica Gel GF254

Mobile phase : Chloroform (97) : Diethyl ether (2.3) : Acetic acid (0.3)

Sample : Alcoholic extract.

Solvent front : 16.4 cm.

Detecting agent : UV detector for TLC at 365nm.

Table: 09 ***TLC Profile of Sterols***

Spot No	Visible Detection (Colour of the Spot)	UV_{365} Detection (Colour of the spot)	Distance traveled (Cm)	R_f
01	Light Green	Violet	03.6	0.22
02	Dark Green	Red	03.9	0.24
03	Light Green	Pink	04.3	0.26
04	---	Pink	04.5	0.27
05	---	Pink	06.2	0.38
06	---	Pink	06.4	0.39
07	---	Violet	09.1	0.56
08	---	Green	12.0	0.73
09	---	Pale Green	13.5	0.82

07. ISOLATION OF PHYTOCONSTITUENTS:

07.1. Isolation of sterols from D*elonix regia* (Boj. ex Hook.) Raf. leaves[81&88]:

100gms of the air-dried plant material was exhaustively extracted using 500ml of Petroleum ether (40 – 60°) in a soxhlet extractor. The solvent was removed under vacuum in a rotary flash evaporator, which leaves a dark green waxy residue. The residue was taken and saponified with alcoholic potassium hydroxide (30gms of potassium hydroxide was dissolved in a minimum quantity of water and the volume was made up to 750ml with alcohol. Allowed to stand over night and the clear supernatant liquid was used) for an hour. Saponified material was then transferred to a separator

and the contents of the flask were washed with about 100 ml of water and run in the separator, contents of the separator were then extracted with successive quantities of petroleum ether 30ml each for 4 times (30 × 4). The ethereal solution was then transferred to a second separator containing 75ml of distilled water, the contents were shaken gently and the aqueous layer was run off. The ethereal solution was then washed by shaking with successive quantities of distilled water (50 × 3). The petroleum ether layer was extracted with 25ml of 0.5N aqueous potassium hydroxide (25 × 2), shaking vigorously in each extraction, later the petroleum ether layer was shaken washed with 25ml of distilled water till the aqueous layer was no longer alkaline to phenolphthalein.

The petroleum ether was recovered under vacuum in a rotary flash evaporator. This is kept overnight in refrigerator yields an amorphous powder of unknown crude sterols which were named as **S_1**, the S_1 sterols are removed by filtration and the pale greenish yellow residue obtained was shaken with small quantity of acetone and filtered. The filtrate was left overnight in a evaporating dish, an amorphous powder of sterols formed at the base of the dish. This is named as **S_2**.

The percentage yield of sterols obtained was 6.0 %w/w.

a. The percentage yield of S_1 sterols was: 1.8% w/w

b. The percentage yield of S_2 sterols was: 4.2 % w/w

The isolated sterols were subjected to phytochemical test to detect the presence of sterols.

07.1.1. Phytochemical tests for Isolated Sterols:

A. Libermann-Buchard test: Isolated compounds are treated with few drops of acetic anhydride, boil and cool, conc. Sulfuric acid is added from the sides of the test tube, shows a brown ring at the junction of two layers and the upper layer turns green which shows the presence of Sterols.

B.Salkowski test: Isolated products were treated with chloroform and few drops of conc. Sulfuric acid, shake well and allow to stand for some time, red color appears at the lower layer indicates the presence of Steroids.

07.1.2. TLC Profile Of Isolated Crude Sterols[87]

Stationary phase : Silica Gel GF254

Mobile phase : Chloroform (97) : Diethyl ether (2.3) : Acetic acid (0.3)

Sample : Isolated sterols.

Solvent front : 13.8 cm.

Detecting agent : UV detector for TLC at 365nm.

Table: 10 ***TLC Profile of Isolated Crude Sterols***

Spot No	Visible Detection (Colour of the Spot)	UV_{365} Detection (Colour of the spot)	Distance traveled (Cm)	R_f
01	Light Green	Violet	03.2	0.23
02	Dark Green	Red	03.4	0.24
03	Light Green	Pink	03.6	0.26
04	---	Pink	05.4	0.39
05	---	Violet	07.7	0.56
06	---	Green	10.1	0.73

7. 2. CHROMATOGRAPHY[89&90]

Chromatography is essentially a technique for the separation of the components of mixtures by a continuous distribution of the components between two phases, one of which is moving past the other. The systems associated with this definition are:

a. A solid stationary phase with a liquid or gaseous mobile phase and

b. A liquid stationary phase with a liquid or gaseous mobile phase; the former

1. Gives rise to adsorption chromatography and the latter

2. To partition chromatography.

The theoretical treatment of these divisions gives results which are not entirely satisfactory and the experimental procedure are diverse. The latter however, have followed from the development of chromatography from its early days and it is convenient to consider the procedures separately.

7.2.1. Column Chromatography

The technique was originally developed by the Russian botanist Tswett in 1906 during the course of an investigation into the nature of leaf pigments. He found that leaf pigments extracted with light petroleum were adsorbed on the top of a column of calcium carbonate supported in a glass tube. As more solvent was allowed to percolate through the column the region of pigmentation became broader and finally separated into distinct and differently coloured bands. Prolonged washing with solvent caused complete separation of the bands which could be eluted separately. It is one of the simplest laboratory exercises to illustrate the use of column chromatography.

The principle underlying the separation of the components is adsorption at the solid liquid interface. For successful separation, the compounds of a mixture must show different degrees of affinity for the solid support (or adsorbent) and the interaction between adsorbent and component must be

reversible. As the adsorbent is washed with fresh solvent the various components will therefore moved down the column until, ultimately, they are arranged in order of their affinity for the adsorbent. Those with least affinity move down the column at a faster rate than those with greater affinity for the adsorbent.

An adsorbent which is already saturated with respect to one substance may take up a small quantity of a second. The latter displaces the former and consequently if a solution of a mixture is percolated continuously through the column and the elute is examined for the presence of substances.

7.2.2. Preparation of column

Prepare the chromatographic column by mixing the adsorbent into a slurry with the solvent and pouring the mixture into the glass tube which contain the solvent. The filtering aid serves to give a flat base to column of adsorbent. After the adsorbent has settled, add a filter paper disc and cotton then run of the supernatant liquid until the level falls to about 1cm above the top layer of cotton. The filter paper disc and cotton is one means of avoiding disturbances of the adsorbent as fresh mobile phase is added to the column in the initial stages of development. The level of solvent must never

be allowed to fall below the level of adsorbent, otherwise the latter develops cracks and becomes useless for chromatography because the solvent runs through the cracks rather than between the particles of adsorbent.

The preparation of slurry may prove difficult with dense adsorbents and it is convenient to pour the powder directly into the solvent in the tube. Frequent tapping of the tube and stirring of the mixture assists in even packing and removal of air bubbles or pockets. Alternatively, the tube may be packed with the dry powder and the solvent allowed to percolated through with the stopcock open until the level falls to about 1 cm above the adsorbent.

The dimension of the column and quantity of adsorbent depend upon the nature and amount of the substance to be chromatographed.

Table: 11 **Adsorbents and Solvents**

S.No	Nature	Adsorbent	Solvents
01	Weak	Sucrose Starch Inulin Talc Sodium carbonate	Petroleum ether Carbon tetrachloride Cyclohexane Carbondisulphide Ether (ethanol free) Acetone Benzene Toluene Esters Chloroform Alcohols Water Pyridine Organic acids
02	Mediu m	Calcium carbonate Calcium phosphate Magnesium carbonate Magnesium oxide Calcium hydroxide	
03	Strong	Activated magnesium silicate Activated alumina Activated charcoal Activated magnesia	

		Fullers earth	Mixture of acids or bases with ethanol or pyridine
04	Neutral	Silica gel	

Table: 12 **Column characteristics**

01	Adsorbent/adsorbate weight ratio	30:01
02	Length/diameter ratio	10-15:01
03	Column length	
	(a) Multi component system	Long column
	(b) Components with similar affinities for adsorbent	Long column
	(c) Components with different affinities for adsorbent	Short column

Note:

* The 30:01 ratio is suitable for preparative separations. For analytical purposes the ratio (30:01) is much too small and often mg quantities of substances are chromatographed on 20g or more adsorbent

* In general , narrow columns give better separations than wide columns.

7.2.3. Use of Column

Wash the column with about 50ml of the mobile phase used to prepare it, which should be the least polar solvent in which the mixture will dissolve. Add the mixture dissolved in a small volume of solvent and carefully allow it run into the filter aid layer by opening the stopcock. Add a small volume of solvent and wash in the mixture. Repeat with gradually increasing quantities of solvent and develop the chromatogram, collecting the eluate in appropriate receivers if the components are to be eluted from the column.

7.2.4. Detection and Recovery of Components

For those mixtures which are coloured visual examination of the column is usually sufficient to locate the coloured components. Colourless components may also be detected visually if they fluoresce, e.g. Quinine and Ergometrine. Recovery of the components after detection on the column

requires extrusion of the column of adsorbent and isolation of each zone for extraction with solvents. If plastic tubing is used instead of glass tubes the zones are conveniently isolated by cutting the tubing into sections.

It is however, more convenient to complete the chromatogram by eluting the various components with solvents. For colourless compounds the eluate is collected as a large number of fractions, each of small volume.

The advent of automatic fraction collectors has enabled large numbers of fractions to be obtained without the tedium associated with manual collection. The large number of fractions also assists in obtaining better separation of components providing attention is directed to correct choice of flow rate.

Each fraction is examined appropriately for the presence of a compound. The examination may be by evaporation of the solvent from each fraction and weighing the residue, by simple spot tests, by examination of the fraction by paper or thin layer chromatography or by spectrophotometry, either directly or after addition of reagents.

7.2.5. ISOLATION OF PHYTOCONSTIUENTS BY COLUMN CHROMATOGRAPHY

Length of the column : 60cm (2 feet)

Diameter : 2.5cm

Stationary phase : Silica gel for Column (60 –120 mesh size)

Mobile phases:

n-Hexane, Petroleum ether 60-80, chloroform, Ethyl acetate & Methanol.

Column Packing Method: Wet packing

The adsorbent silica gel is made into a slurry with n-Hexane and poured inside the column and the adsorbent is allowed to settle down. The column is packed by this method for about a length of 30-40 cm.

Sample preparation:

About 15gms of the leaf alcoholic extract is taken and dissolved in hexane and slurred with silica gel 60 – 120 and it was transferred into the column.

The contents were then covered by means of cotton plugging.

Elution rate : 01 drop/sec

The contents of the column were then eluted using organic solvents of following proportions:

Table: 13 Column Chromatography for phytoconstituents isolation

S.No	Mobile Phase	Proportion %	Quantity utilized	Number of fractions collected
01	N -Hexane	100	300ml	01 – 15
02	N-Hexane : Petroleum ether	90:10	300ml	16 - 30
03	N – Hexane: Petroleum ether	80:20	300ml	31 – 40
04	N – Hexane: Petroleum ether	70:30	300ml	41 – 50
05	N – Hexane : Petroleum ether	60:40	300ml	51 – 60
06	N – Hexane : Petroleum ether	50:50	300ml	61 – 70
07	Petroleum ether	100	300ml	71 – 82
08	Petroleum ether: Chloroform	90:10	300ml	83 – 93
09	Petroleum ether:	80:20	300ml	94 – 104

	Chloroform Petroleum			
10	**ether:**	**70:30**	**300ml**	**105 – 114**
	Chloroform Petroleum			
11	**ether:**	**60:40**	**300ml**	**115 – 125**
	Chloroform Petroleum			
12	**ether:**	**50:50**	**500ml**	**126 - 159**
	Chloroform			
13	**Chloroform**	**100**	**300ml**	**160 – 172**
	Chloroform:			**173 – 185**
14		**90:10**	**300ml**	
	Ethyl acetate			
	Chloroform:			
15		**80:20**	**300ml**	**186 – 196**
	Ethyl acetate			
	Chloroform:			
16		**70:30**	**300ml**	**197 – 207**
	Ethyl acetate			
	Chloroform:			
17		**60:40**	**300ml**	**208 – 221**
	Ethyl acetate			
	Chloroform:			
18		**50:50**	**300ml**	**222 – 234**
	Ethyl acetate			
19	**Ethyl acetate**	**100**	**300ml**	**235 – 247**
	Ethyl acetate:			
20		**90:10**	**300ml**	**248 – 265**
	Methanol			
	Ethyl acetate:			
21		**80:20**	**300ml**	**266 – 277**
	Methanol			
22	**Ethyl acetate:**	**70:30**	**300ml**	**278 – 290**

23	**Methanol Ethyl acetate: Methanol**	**60:40**	**300ml**	**291 – 303**
24	**Methanol Ethyl acetate: Methanol**	**50:50**	**300ml**	**304 – 316**
25	**Methanol**	**100**	**400ml**	**317 - 335**

After collection of each fraction, they were subjected to TLC using Silica Gel GF 254 using the same mobile phase of their elution and observed under UV 254/365 and based upon the nature preliminary phytochemical tests and specified TLC profiles were carried out.

Table: 14 Detection of Phytoconstituents from various fractions

S.No	Fractions	Mobile Phase	Number of Spots
01	01 – 15	N –Hexane 100%	03
02	16 - 30	N - Hexane 90%: Petroleum ether 10%	02
03	31 – 40	N – Hexane 80%: Petroleum ether 20%	01 (S_3)
04	41 – 50	N – Hexane 70%: Petroleum ether 30%	Nil
05	51 – 60	N – Hexane 60%: Petroleum ether 40%	02

06	61 – 70	N – Hexane 50%: Petroleum ether 50%	01 (S_4)
07	71 – 82	Petroleum ether 100%	01 (S_4)
08	83 – 93	Petroleum ether 90%: Chloroform 10%	Nil
09	94 – 104	Petroleum ether 80%:Chloroform 20%	01 (S_5)
10	105 – 114	Petroleum ether 70%:Chloroform 30%	01 (S_6)
11	115 – 125	Petroleum ether 60%:Chloroform 40%	01 (S_7)
12	126 - 159	Petroleum ether 50%: Chloroform50%	01 (S_8)
13	160 – 172	Chloroform 100%	06
14	173 – 185	Chloroform 90% : Ethyl acetate 10%	06
15	186 – 196	Chloroform 80%: Ethyl acetate 20%	04
16	197 – 207	Chloroform 70%: Ethyl acetate 30%	Nil
17	208 – 221	Chloroform 60%: Ethyl acetate 40%	Nil
18	222 – 234	Chloroform 50%: Ethyl acetate 50%	04
19	235 – 247	Ethyl acetate 100%	03
20	248 – 265	Ethyl acetate 90%: Methanol 10%	01 (F_1)
21	266 – 277	Ethyl acetate 80%: Methanol 20%	Nil
22	278 – 290	Ethyl acetate 70%: Methanol 30%	Nil
23	291 – 303	Ethyl acetate 60%: Methanol 40%	Nil
24	304 – 316	Ethyl acetate 50%: Methanol 50%	Nil
25	317 - 335	Methanol 100%	Nil

Identification of isolated phytoconstituents

01. S_3 .

Stationary Phase : Silica Gel GF254

Mobile Phase : n – Hexane (80) : Petroleum ether (20)

Detector : UV 254/365

Colour : Fluorescent Green

R_f : 0.50

Quantity collected : 14mg

Collection procedure: The fractions (31-40) which are shown the same R_f, colour with respect to a particular mobile phase with fixed proportions are pooled together and evaporated to dryness and collected.

02. S_4 .

Stationary Phase : Silica Gel GF254

Mobile Phase : n – Hexane (50) : Petroleum ether (50)

Detector : UV 254/365

Colour : Fluorescent Green

R_f : 0.40

Quantity collected : 16mg

Collection procedure: The fractions (61-82) which are shown the same R_f, colour with respect to a particular mobile phase with fixed proportions are pooled together and evaporated to dryness and collected.

03. S_5 .

Stationary Phase : Silica Gel GF254

Mobile Phase : Petroleum ether (80) : Chloroform (20)

Detector : UV 254/365

Colour : Light Green

R_f : 0.45

Quantity collected : 26.2mg

Collection procedure: The fractions (94-104) which are shown the same R_f, colour with respect to a particular mobile phase with fixed proportions are pooled together and evaporated to dryness and collected.

04. S_6 .

Stationary Phase : Silica Gel GF254

Mobile Phase : Petroleum ether (70) : Chloroform (30)

Detector : UV 254/365

Colour : Fluorescent yellow

R_f : 0.75

Quantity collected : 07mg

Collection procedure: The fractions (105 – 114) which are shown the same R_f, colour with respect to a particular mobile phase with fixed proportions are pooled together and evaporated to dryness and collected.

---

05. S_7 .

Stationary Phase : Silica Gel GF254

Mobile Phase : Petroleum ether (60) : Chloroform (40)

Detector : UV 254/365

Colour : Yellow

R_f : 0.70

Quantity collected : 27mg

Collection procedure: The fractions (115-125) which are shown the same R_f, colour with respect to a particular mobile phase with fixed proportions are pooled together and evaporated to dryness and collected.

06. S_8 .

Stationary Phase : Silica Gel GF254

Mobile Phase : Petroleum ether (50) : Chloroform (50)

Detector : UV 254/365

Colour : Fluorescent green

R_f : 0.34

Quantity collected : 473mg

Collection procedure: The fractions (126-159) which are shown the same R_f, colour with respect to a particular mobile phase with fixed proportions are pooled together and evaporated to dryness and collected.

07. F_1 .

Stationary Phase : Silica Gel GF254

Mobile Phase : Ethyl acetate (9) : Methanol (1)

Detector : UV 254/365

Colour : Green

R_f : 0.85

Quantity collected : 100mg

Collection procedure: The fractions (248-265) which are shown the same R_f, colour with respect to a particular mobile phase with fixed proportions are pooled together and evaporated to dryness and collected.

08. S_1 .

Stationary Phase : Silica Gel GF254

Mobile Phase : Chloroform (97): Diethyl ether(2.3) : Conc. Acetic acid (0.5)

Detector : UV 254/365

Colour : Light rose

R_f : 0.25

09. S_2 .

Stationary Phase : Silica Gel GF254

Mobile Phase : Chloroform (97): Diethyl ether(2.3) : Conc. Acetic acid (0.5)

Detector : UV 254/365

Colour : Light rose, pink, Green and Flourescent yellow.

R_f : 0.25, 0.4, 0.6 & 0.88

Each isolated sterols (S_3 –S_8) were first subjected to preliminary phytochemical tests of Libberman Buchard and Salkowski tests and further confirmed by means of carrying out the specific TLC profiles for sterols using Chloroform (97): Diethyl ether(2.3) : Conc. Acetic acid (0.5) as the mobile phase.

In the similar way the isolated flavanoid F_1 was subjected to preliminary phytochemical tests of Shinoda test (Magnesium Hydrochloride reduction test), Zinc Hydrochloride reduction test and Alkaline reagent tests and further confirmed by means of carrying out the specific TLC profiles for flavanoids using Benzene (9) : Acetone (1) as the solvent system.

PLAN OF THE WORK

- Collection, Authentication & Shade drying of ***Delonix regia*** (Boj. ex Hook.) Raf. leaves

- Coarse powdering of the air dried plant material.

- Extraction of the material using 95% ethanol.

- Preliminary phytochemical screening of plant extracts.

- Preliminary TLC studies of the plant extracts.

- Crude isolation of sterols.

- Isolation of phytoconstituents by Column chromatography.

- Confirmation of the isolated compounds by means of phytochemical and TLC studies.

- ✯ Spectral elucidation of the isolated compounds through
  - ► UV
  - ► IR
  - ► NMR
  - ► GC-MS

- ✯ Determination of impurities present in the isolated compound by HPLC technique.

- ✯ Structural elucidation of the isolated compounds.

- ✯ Evaluation of Anti-microbial potential of the crude extract.

- ✯ Determination of Anti-oxidant potential of isolated compounds by
  - ❖ Estimation of Total phenolics as a preliminary measure of antioxidant potential.

- ❖ In vitro models
  - ► Reducing power ability
  - ► DPPH radical scavenging assay
  - ► Nitric Oxide radical scavenging assay.

- ❖ Ex vivo model
  - ► Estimation of TBAR's (Lipid peroxidation assay)

- ✯ Statistical analysis.

ANALYTICAL STUDIES

Analytical chemistry deals with methods for determining the chemical composition of samples of matter. A qualitative methods yields information about the identity of atomic or molecular species or the functional groups in the sample. A quantitative method in contrast provides numerical information as to the relative amount of one or more of these components.

In the early years of chemistry, most analyses were carried out by separating the components of interest in a sample by precipitation, extraction or distillation. Early in the 20^{th} century, chemists began to exploit phenomenon other than those used for classical methods for solving analytical problems. Thus measurements of physical properties of analytes such as conductivity, electrode potential, light absorption or emission, mass to charge ratio and fluorescence began to be used for quantitative analysis of variety of inorganic, organic and biochemical analytes.

Furthermore, highly efficient chromatographic and electrophoretic technique began to replace distillation, extraction and precipitation for the separation of components of complex mixtures prior to their qualitative or quantet

determination. These newer methods for separating and determining chemical species are known collectively as instrumental methods of analysis has paralleled the development of the electronic and computer industries.

1. FLAME EMISSION SPECTROSCOPY[91&92]

The absorption and emission of radiant energy by atoms provide powerful analytical tools for both quantitative and qualitative analysis. Most recently, new sources for plasma emission spectroscopy offer capabilities this complement Flame Emission Spectroscopy and Atomic Absorption Spectroscopy for many analysis.

Flame photometry is also named as FLAME EMISSION SPECTROSCOPY because of the use of a flame to provide the energy of excitation to atoms introduced into the flame.

Flame photometry is based on the measurement of intensity of the light emitted when a metal is introduced into a flame. The wavelength of the colour tells us what the element is and the colours intensity tells us how much of the element is present.

The instrument permitted us to select the wavelength of the radiation and measure its intensity with considerable accuracy. The spectrophotmetric technique has proven to be one of most reliable and easily used techniques for the determination of concentration of sodium, potassium, calcium and magnesium.

Flame photometry coupled with simple read-out devices, provides high sensitivity and high reliability for the determination of elements in the first two columns of the periodic table. Among these elements are sodium, potassium, lithium, calcium, magnesium, strontium and barium. The measurement of these elements is very useful in medicine, agriculture and plant science. Flame photometry is also successful in determining certain transition elements such as copper, iron and manganese.

Flame photometry is a simple, rapid method fro the routine determination of elements that can be easily excited.

1.1. Applications

* The most useful application of flame photometry is for the rapid quantitative determination of the elements in the group I and II of the periodic table, using equipment with high optical resolution; other metallic elements may also be determined.

* The principal analytical advantages of flame photometry include its simplicity and speed.

* Flame photometry gives no information on molecular analysis, but it is used widely for elemental analysis.

1.2. Determination of Na^+, K^+ and Ca^{2+} in Delonix regia leaves

This determination is based on a flame photometry method utilizing atomic absorption (AA). The liquid sample containing sodium, potassium and calcium ions is aspirated into a very well-defined and shaped flame. The ions are reduced in the flame to atoms. Light from a lamp passes through the flame and is absorbed specifically by the selected atoms in the flame. With a constant aspiration rate (flow), and consistent formation of sample aerosol droplets in the flame, the concentration of Na^+, K^+ and Ca^{2+} atoms in the

flame is proportional to the concentration in the liquid sample. Absorption of the light from the lamp by the selected atoms in the flame follows Beer's Law. The instrument obtains the flame absorbance of a sample by comparing the transmittance (P) with that of a blank solution (Po).

$A = \log[Po/P] = abC(unk)$

The method used to obtain the ionic concentration is the standard addition approach used with solution photometry.

1.2.1. Preparation of Solutions

Results of the solution photometry experiment should provide an idea of the ions concentration. Convert the concentration to ppm of Na^+, K^+ and Ca^{2+}.

Stock solution:

* Sodium = 50 mEq/L (as Na^+)

Dissolve 2.923 gm of NaCl in one litre of distilled water.

* Potassium = 50 mEq/L (as K^+)

Dissolve 3.728 gm of KCl in one litre of distilled water.

* Calcium = 50 mEq/L (as Ca^{2+})

Dissolve 2.503 gm of $CaCO_3$ in approximately 300ml of distilled water and 10ml of Conc.Hcl, Dilute to one litre with water.

Dilutions

* For Na^+

0.5ml to 100ml, which is equivalent to 1 mEq.

1.0ml to 100ml, which is equivalent to 2 mEq.

2.0ml to 100ml, which is equivalent to 4 mEq.

* For K^+

1.0 ml to 100ml, which is equivalent to 0.1 mEq.

10 ml to 100ml, which is equivalent to 1.0 mEq.

20ml to 100ml, which is equivalent to 2.0 mEq.

* For Ca^{2+}

5.0ml to 100ml, which is equivalent to 2.5 mEq.

10ml to 100ml, which is equivalent to 5.0 mEq.

20ml to 100ml, which is equivalent to 10 mEq.

1.2.2. Sample preparation:

One gm of the powdered drug is taken and total ash of the same is determined using muffle furnace. The yield of total ash was found to be 07.3967%. The obtained ash is chopped with 5% Hydrochloric acid (100ml). The contents were mixed properly and filtered. The filtrate is taken and made up to 100ml with water. The sample is taken for elemental analysis.

1.2.3. Procedure

The flame photometer is calibrated for Na^{+}, K^{+} and Ca^{2}+ ions separately using the diluted stock solutions. After the calibration of the instrument the prepared sample is taken for analysis separately for each ions.

The blank solution used for the AA measurements can be simply deionized water.

The obtained mEq values are converted to ppm by multiplying the value with molecular weight of the substance.

Table: 15 **Content of Na^+, K^+ and Ca^{2+} in *Delonix regia* (Boj. ex Hook.) leaves**

S.No	Ions	mEq	ppm
01	**Sodium (Na^+)**	0.5	11.4949
02	**Potassium (K^+)**	0.1	03.9098
03	**Calcium (Ca^{2+})**	1.0	10.0800

2. SPECTROMETRIC METHODS[93-99]

These are of 3 types:

a. Molecular spectrometry

b. Mass spectrometry

c. X-ray spectrometry

2.1. Molecular spectrometry

The molecular spectrometry comprises of UV/Visible, IR and NMR spectroscopy. In which the UV is based upon absorption, IR on Vibration and NMR by means of Spinning.

2.2. Mass spectrometry

In atomic mass spectrometry, samples are also atomized, but in this case, the gaseous atoms are converted to positive ions (usually single charged) and

separated on the basis of their mass to charge ratios. Quantitative data are then obtained by counting the separated ions.

2.3. X-ray spectrometry

Atomization is not required because X-ray spectra for most elements are largely independent of how they are chemically combined in sample.

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3. 0 Spectroscopy:

3.1. ULTRA VIOLET AND VISIBLE SPECTROSCOPY

3.1.1. Principle:

When UV or visible radiation is passed through a substance under examination, absorption of energy results in the promotion of electron from the ground electronic state to the excited electronic state. During the process of absorption, a large number of photon-molecule collisions are possible but only those collisions will cause absorption of energy in which the energy of photon matches the energy difference between the ground and excited electronic state of the molecule. The absorption of energy is quantised. In UV and Visible spectroscopy, electronic transition takes place.

- UV is the electronic spectroscopy since it involves the promotion of electrons (σ, π, n* electrons) from the ground state to the higher energy state.
- It is very useful to measure the number of conjugated double bonds and also aromatic conjugation within the various molecule.

- It also distinguish between conjugated and non conjugated system α,β- unsaturated carbonyl compounds from β, γ analogues, homo annular, hetero annular conjugated dienes etc.,
- For visible and UV spectrum, electronic excitations occur in 200-800 nm and involves the promotion of electrons to the higher energy molecular orbital.
- There are two laws with govern the absorption of light by the molecules. These are Lambert's law and Beers law.
- Ultra-Violet spectroscopy has been mainly applied for the detection of functional group (Chromophore), the extent of conjugation, detection of polynuclear compounds by comparison.
- Electronic spectroscopy : UV 200-400 nm.

Visible 400 – 800 nm.

3.2. **INFRA RED SPECTROSCOPY**

Infra red spectrum is an important about the structure of a compound. This technique provides a spectrum containing a large number of absorption bands from which a wealth of information can be derived about the structure of an organic compound.

The IR region of the spectrum encompasses radiation with wave numbers ranging from about 12,800 – 10 Cm^{-1} or wave length from 0.78 to 1000μm. From the stand point of both application and instrumentation, the IR spectrum is conveniently divided into near, mid and far Infra Red radiation (Rough limitations of).

The technique can be employed to establish the identity of two compounds or to determine the structure of a new compound. Its quite useful to predict the presence of certain fundamental groups which absorps at definite frequencies, for example the hydroxyl group in a compound absorps at 3600 – 3200 Cm^{-1} , carbonyl group of ketone groups give a strong band at 1710 Cm^{-1} . It is thus a reliable technique for disclosing the identity of a compound.

* It has been found that, no two compounds except the enantiomers can have similar Infra-red spectra.

The IR spectrum of a compound provide more information than is normally available from the electronic spectra.

In this technique, almost all groups absorb characteristically with in a definite range. The shift in the position of absorbing for a particular group may change with the changes in the structure of the molecule.

Impurities in a compound can be detected from the nature of the bands, which no longer remain and well defined. Example, if the spectrum contains a strong absorption band between 1,900 – 1,600 Cm^{-1} the presence of carbonyl group (-C=O) in a compound is suspected.

The position of the peak or the band not only tells the presence of a particular group but also reveals a good deal about the environment affecting the group.

3.3. NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

NMR spectroscopy has become a powerful tool for the organic chemist. This technique is only applicable to those nuclei which possess a spin quantum number greater than zero. The two types of spectra most frequently encountered by the organic chemist are ^{1}H and ^{13}C NMR spectra.

3.3.1. Proton NMR Spectroscopy

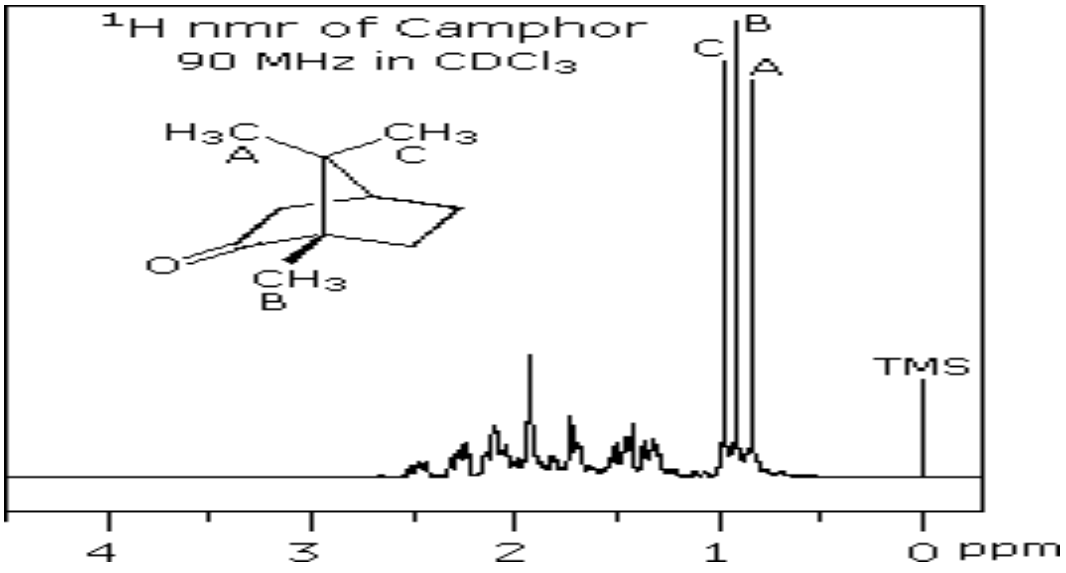
This important and well-established application of nuclear magnetic resonance will serve to illustrate some of the novel aspects of this method. To begin with, the NMR spectrometer must be tuned to a specific nucleus, in this case the proton. The actual procedure for obtaining the spectrum varies, but the simplest is referred to as the continuous wave (CW) method. A solution of the sample in a uniform 5 mm glass tube is oriented between the poles of a powerful magnet, and is spun to average any magnetic field variations, as well as tube imperfections. Radio frequency radiation of appropriate energy is broadcast into the sample from an antenna coil (colored red). A receiver coil surrounds the sample tube, and emission of

absorbed rf energy is monitored by dedicated electronic devices and a computer.

An NMR spectrum is acquired by varying or sweeping the magnetic field over a small range while observing the rf signal from the sample. An equally effective technique is to vary the frequency of the rf radiation while holding the external field constant. For example Proton NMR spectra of camphor is

3.3.2. ^{1}H NMR OF CAMPHOR

Spectra: 01



3. 3. 3. Carbon NMR Spectroscopy

The power and usefulness of ^{1}H NMR spectroscopy as a tool for structural analysis should be evident from the past discussion. Unfortunately, when significant portions of a molecule lack C-H bonds, no information is forthcoming.

Even when numerous C-H groups are present, an unambiguous interpretation of a proton NMR spectrum may not be possible.

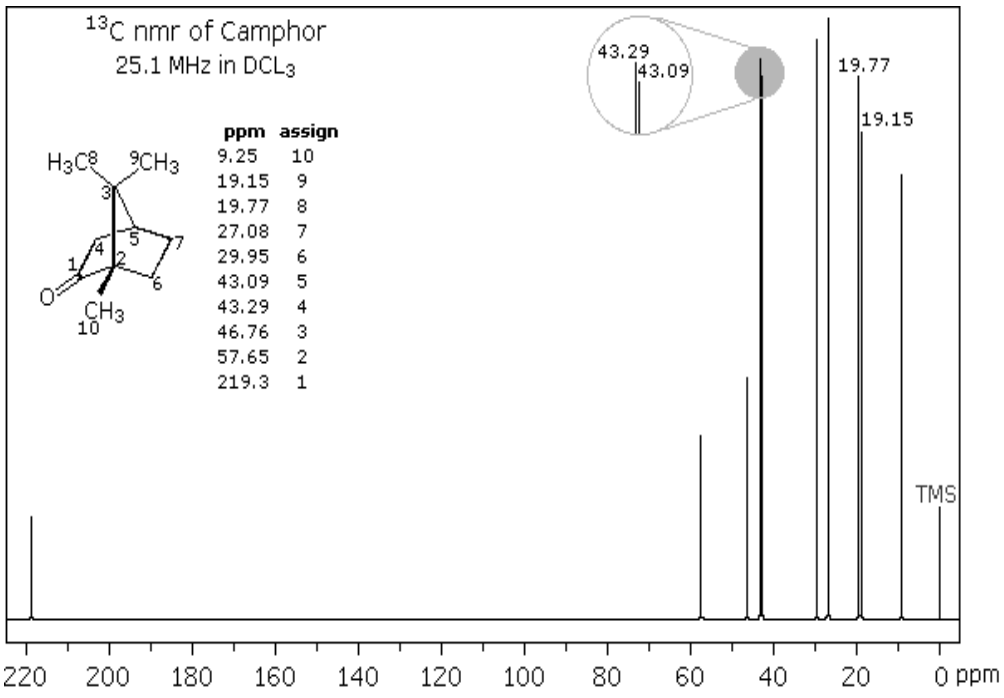
These difficulties would be largely resolved if the carbon atoms of a molecule could be probed by NMR in the same fashion as the hydrogen atoms. Since the major isotope of carbon (^{12}C) has no spin, this option seems unrealistic. Fortunately, 1.1% of elemental carbon is the ^{13}C isotope, which has a spin I = 1/2, so in principle it should be possible to conduct a carbon NMR experiment. It is worth noting here, that if much higher abundances of ^{13}C were naturally present in all carbon compounds, proton NMR would become much more complicated due to large one-bond coupling of ^{13}C and ^{1}H.

The most important operational technique that has led to successful and routine ^{13}C NMR spectroscopy is the use of high-field pulse technology coupled with broad-band hetero nuclear decoupling of all protons. The results of repeated pulse sequences are accumulated to provide improved signal strength. Also, for reasons that go beyond the present treatment, the decoupling irradiation enhances the sensitivity of carbon nuclei bonded to hydrogen. When acquired in this manner, the carbon NMR spectrum of a compound displays a single sharp signal for each structurally distinct carbon atom in a molecule (remember, the proton couplings have been removed). The spectrum of camphor, shown on the left below, is typical. Furthermore, a comparison with the ^{1}H NMR spectrum on the right illustrates some of the advantageous characteristics of carbon NMR. The dispersion of ^{13}C chemical shifts is nearly twenty times greater than that for protons, and this together with the lack of signal splitting makes it more likely that every structurally distinct carbon atom will produce a separate signal. The only clearly identifiable signals in the proton spectrum are those from the methyl groups. The remaining protons have resonance signals between 1.0 and 2.8 ppm from TMS, and they overlap badly thanks to spin-spin splitting. For example

13C NMR of Camphor is as follows

3.3.4. ^{13}C NMR OF CAMPHOR

Spectra : 02



Unlike proton NMR spectroscopy, the relative strength of carbon NMR signals are not normally proportional to the number of atoms generating each one. Because of this, the number of discrete signals and their chemical shifts are the most important pieces of evidence delivered by a carbon spectrum.

3.4. MASS SPECTROMETRY

Mass spectrometry is perhaps the most widely applicable of all analytical tools provides qualitative and quantitative information about the atomic and molecular composition of organic and inorganic materials, biological matter.

- The elemental composition of samples of matter.
- The structure of organic, inorganic and biological molecules.
- Qualitative and quantitative composition of complex mixtures.
- The structure and composition of solid surfaces.
- Isotopic ratios of atoms in samples.

In this technique, molecules are bombarded with a beam of energetic electrons. The molecules are ionized and broken up into many fragments, some of which are positive ions. Each kind of ion has a particular ratio of mass to charge ratio.

Mass spectrum is a record of the masses and the relative abundances of the molecular ion and the positively charged fragments formed from it by the electron bombardment. The mass spectrum of a pure compound provides several kind of data which are useful for its identification.

The mass spectrum is always recorded in the gaseous state. It means that complications due to solvents and change of physical state don't affect the nature of mass spectrum.

In mass spectrometry, the quantity of the sample required designated as M+. It is positively charged molecule with unpaired electrons. The set of fragment or daughter ions are analysed in such a way that a signal is obtained for each volume m/e i.e. is represented. The intensity of each signal represents the relative abundance of the ion producing the signal. The largest peak in the structure is called base peak and its intensity is taken as 100. The intensities of other peaks are represented relative to the base peak. The fragmentation or cracking pattern for a single substance is uniquely characteristic and may be used for qualitative identification purposes.

4. CHROMATOGRAPHIC TECHNIQUES

Analytical separations were largely carried out by such classical methods as precipitation, distillation and extraction. By now, however analytical separations are most commonly carried out by chromatography and electrophoresis, particularly with samples that are multicomponent and complex. There are various types of chromatographies are there one among them is High Performance Liquid Chromatography (HPLC).

4.1. HIGH PERFORMANCE LIQUID CHROAMTOGRAPHY (HPLC)

The technique of high performance liquid chromatography was developed in the late 1960s and early 1970s from a knowledge of the theoretical principles that already had been established for the earlier chromatography techniques, in particular for column chromatography and from advances made in column packing materials and in the design of chromatographic equipment.

HPLC ranks among the most widely used techniques in pharmaceutical analysis. This is due to several reasons:

- Wide variety of packing materials allows to separate of most chemical species.
- Different types of detectors available permit the sensitive detection of most chemical types, accuracy and precision with which eluted substances may be quantified give analytical data of the highest caliber.
- Micro particulate packing materials give excellent separation of similar substances.
- Short columns in routine use allow fast separation to take place and complete separation of a complex mixtures can be achieved within a few minutes.
- Use of automatic samples and injectors enables large number of samples to be analysed unattended.
- Combination of HPLC and spectrometric techniques allows the almost simultaneously quantitation and identification of solutes as they elute from the column.

- Principal areas of pharmaceutical analysis in which HPLC is routinely used are the quality control testing of drugs and medicines for compliance with specifications, stability studies, therapeutic monitoring, drug metabolism studies and pharmacokinetic studies.
- Useful in providing compound specific assays.
- Separation and control of impurities.
- Column may be reused.
- Applied to wide variety of natural products such as nucleic acids, urine, serum, carbohydrates, lipids, amino acids, bile acids, pharmaceutical products, pesticides, herbicides, surfactants and antioxidants.
- Reverse phase partition HPLC is particularly useful for the separation of polar compounds such as drug metabolites, peptides, vitamins, poly phenols and steroids.

- In principle HPLC arose from conventional liquid column chromatography, following the development of gas liquid chromatography and realization that it was a rapid and accurate analytical method.

5.0 Spectral and HPLC studies of the isolated compounds.

The isolated compounds were subjected to various spectral studies and HPLC chromatographic studies as follows.

5. 1. Isolated compound S_1

- COMPOUND CODE:**S_1**

- PHYSICAL EXAMINATION:
  - **Colour:** Light yellow
  - **State :** Amorphous powder

- IDENTIFCATION

a) Chemical tests :Libbermann-Buchard and Salkowski tests.

b) THIN LAYER CHROMATOGRAPHY:

- **Stationary phase :** Silica -gel GF 254
- **Mobile phase** **:**Chloroform(97):Diethyl ether (2.3): Glacial Acetic acid(0.5)
- **Detector** **:** UV 254/365
- **Colour** **:** Light green fluorescence
- **R_f value** **:** 0.25

❖ PHYSICAL CONSTANT

Melting Point: 143℃

❖ SOLUBILITY:

n- Hexane, Petroleum ether, Chloroform, Benzene and Methanol.

❖ YIELD: **1.8 gms**

❖ UV SPECTRAL DATA:

λmax 275nm

❖ **IR SPECTRAL DATA:**

Table: 16 **IR Spectral Details of isolated sterol S_1**

S. No	FREQUENCY cm-1	STRETCHING & DEFORMATION
01	3368	-OH STRETCHING
02	2916-2848	-C-H STRETCHING
03	2360	-C=C- STRETCHING
04	1473-1463	-C-H- DEFORMATION; -C=C- STRETCHING
05	1261	-O-H DEFORMATION ;–C-O STRETCHING
06	1060	Primary OH STRETCHING
07	803-716	Aromatic Substitution

❖ **NMR SPECTRAL DATA: PMR**

Table: 17 **PMR Spectral Details of isolated sterol S_1**

S.NO	SIGNALS (δ) Values ppm	GROUP ASSIGNED
01	7.2	Aryl Protons
02	3.6	Hydroxyl Protons
03	2.2	Aromatic Carbonyl Protons
04	1.2	Secondary Alkyl Protons
05	5.1	Aromatic Protons
06	-0.018	Off the Scale

5. 2. Isolated compound S_2

- COMPOUND CODE : **S_2**

- PHYSICAL EXAMINATION:
  - **Colour :** Reddish orange.
  - **State :** Sticky mass.

- IDENTIFCATION

a)Test : Libermann-Buchard and Salkowski tests.

b) THIN LAYER CHROMATOGRAPHY:

- **Stationary phase :** Silica gel GF 254
- **Mobile phase :**Chloroform(97):Diethyl ether(2.3):Glacial Acetic acid(0.5)
- **Detector :**UV 254/365
- **Colour :**Light Rose, Pink, Green and Flourescent yellow
- **R_f value :** 0.25, 0.4, 0.6, 0.88

- PHYSICAL CONSTANT
  - ✓ **Melting Point:** 138℃

- SOLUBILITY:
  - ✓ n- Hexane, Petroleum ether, Chloroform, Benzene and Methanol.

- YIELD: **4.2 gms.**

- UV SPECTRAL DATA:

  λmax 258 nm

- **IR SPECTRAL DATA:**

Table: 18 **IR Spectral Details of isolated sterol S_2**

S.No	FREQUENCY cm-1	STRETCHING & DEFORMATION
01	3423	-OH STRETCHING
02	2956-2847	C-H STRETCHING
03	1735	C=O STRETCHING in STEROIDAL ESTER
04	1652	C=O STRETCHING
05	1471-1462	-C-H DEFORMATION
06	1378	-C=O-O :Carboxlyate anion Stretching
07	1178	PhenolicO-H STRETCHING &C-O DEFORMATION
08	1060	Primary OH STRETCHING
09	887-719	Aromatic Substitution

5. 3. Isolated compound S_3

- COMPOUND CODE: **S_3**
- PHYSICAL EXAMINATION:
  - **Colour:** White
  - **State :** Waxy
- IDENTIFCATION

a) Chemical tests :Libermann-Buchard and Salkowski tests.

b) THIN LAYER CHROMATOGRAPHY:

- **Stationary phase :** Silica -gel GF 254
- **Mobile phase** **:** Chloroform(97):Diethyl ether (2.3): Glacial Acetic acid(0.5)
- **Detector** **:** UV 254/365
- **Colour** **:** Flourescent green.
- **R_f value** **:** 0.45

❖ PHYSICAL CONSTANT

Melting Point: 148℃

❖ SOLUBILITY:

n- Hexane, Petroleum ether, Chloroform, Benzene and Methanol.

❖ YIELD: **14 mg**

❖ UV SPECTRAL DATA:

λmax **245nm**

5. 4. Isolated compound S_4

- COMPOUND CODE:**S_4**

- PHYSICAL EXAMINATION:

  - **Colour:** White
  - **State :** Waxy

- IDENTIFCATION

a) Chemical tests :Libermann-Buchard and Salkowski tests.

b) THIN LAYER CHROMATOGRAPHY:

- **Stationary phase :** Silica -gel GF 254
- **Mobile phase :**Chloroform(97):Diethyl ether (2.3): Glacial Acetic acid(0.5)
- **Detector :** UV 254/365
- **Colour :** Flourescent yellow.
- **R_f value :** 0.62

- PHYSICAL CONSTANT

  Melting Point: 140℃

- SOLUBILITY:

  n- Hexane, Petroleum ether, Chloroform, Benzene and Methanol.

- YIELD: **16 mg**

- UV SPECTRAL DATA:

  λmax **240nm**

5. 5. Isolated compound S_5

- COMPOUND CODE:**S_5**

- PHYSICAL EXAMINATION:
  - **Colour:** White
  - **State :** Waxy

- IDENTIFCATION

a) Chemical tests

Libermann-Buchard and Salkowski tests.

b) THIN LAYER CHROMATOGRAPHY:

- **Stationary phase :** Silica -gel GF 254
- **Mobile phase :**Chloroform(97):Diethyl ether (23): Glacial Acetic acid(0.5)
- **Detector :** UV 254/365
- **Colour :** Light green.
- **R_f value :** 0.5

♦ PHYSICAL CONSTANT

Melting Point: 165℃

♦ SOLUBILITY:

n- Hexane, Petroleum ether Chloroform & Benzene.

♦ YIELD: **26.2 mg**

♦ UV SPECTRAL DATA:

λmax 241nm

♦ **IR SPECTRAL DATA:**

Table: 19 **IR Spectral Details of isolated sterol S_5**

S.No	FREQUENCY cm-1	STRETCHING & DEFORMATION
01	3415	OH STRETCHING
02	2953-2847	C-H STRETCHING & C=C aromatic STRETCHING
03	1734	C=O STRETCHING in STEROID ESTER
04	1465	-C-C STRETCHING
05	1374	-C-H DEFORMATION
06	1169	-OH DEFORMATION: -C-O STRETCHING Secondary alcohol
07	758-723	Aromatic Substitution

5. 6. Isolated compound S_6

✫ COMPOUND CODE:**S_6**

✫ PHYSICAL EXAMINATION:

- **Colour:** Pale yellow
- **State :** Waxy

✫ IDENTIFCATION

a) Chemical tests :Libermann-Buchard and Salkowski tests.

b) THIN LAYER CHROMATOGRAPHY:

- **Stationary phase :** Silica -gel GF 254
- **Mobile phase :**Chloroform(97):Diethyl ether (2.3): Glacial Acetic acid(0.5)
- **Detector :** UV 254/365
- **Colour :** Flourescent Yellow.
- **R_f value :** 0.81

☆ PHYSICAL CONSTANT

Melting Point: 185℃

☆ SOLUBILITY:

n- Hexane, Petroleum ether Chloroform & Benzene.

☆ YIELD: **07 mg**

☆ UV SPECTRAL DATA:

λmax 253nm

5. 7. Isolated compound S_7

- ► COMPOUND CODE:**S_7**
- ► PHYSICAL EXAMINATION:

  - o **Colour:** Yellow
  - o **State :** Waxy

- ► IDENTIFCATION

a) Chemical tests :Libermann-Buchard and Salkowski tests.

b) THIN LAYER CHROMATOGRAPHY:

- ❖ **Stationary phase :** Silica -gel GF 254
- ❖ **Mobile phase :**Chloroform(97):Diethyl ether (2.3): Glacial Acetic acid(0.5)
- ❖ **Detector :** UV 254/365
- ❖ **Colour :** Yellow.
- ❖ **R_f value :** 0.64

- ► PHYSICAL CONSTANT

Melting Point: 141℃

- SOLUBILITY:

  n- Hexane, Petroleum ether Chloroform & Benzene.

- YIELD: 27 **mg**

- UV SPECTRAL DATA:

  λmax 254nm

- **IR SPECTRAL DATA:**

Table: 20 **IR Spectral Details of isolated sterol S_7**

S.No	FREQUENCY cm-1	STRETCHING & DEFORMATION
01	2919-2850	-C-H STRETCHING
02	2362	-C=C- STRETCHING
03	1735	-C=O STRETCHING IN STERIOD ESTER
04	1375	-C-NO_2 Carboxylate anion Stretching
05	1278	-C-O STRETCHING &–O-H DEFORMATION
06	1094	Secondary OH STRETCHING
07	974-723	Aromatic Substitution

5. 8. Isolated compound S_8

- COMPOUND CODE : **S_8**

- PHYSICAL EXAMINATION:
  - **Colour** **:** Pale yellow
  - **State** **:** Amorphous powder.

- IDENTIFCATION

a)Test **:** Libermann-Buchard and Salkowski tests.

b) THIN LAYER CHROMATOGRAPHY:

- **Stationary phase** **:** Silica gel GF 254
- **Mobile phase** **:**Chloroform(97):Diethyl ether(2.3):Glacial Acetic acid(0.5)
- **Detector** **:**UV 254/365
- **Colour** **:**Green.
- **R_f value** **:** 0.78

- PHYSICAL CONSTANT
  - **Melting Point:** 142℃

- SOLUBILITY:
  - n- Hexane, Petroleum ether, Chloroform & Benzene.

- YIELD: **473mg.**

- UV SPECTRAL DATA:

λmax **275 nm**

- **HPLC Spectra of isolated sterol S_8**

The isolated sterol S_8 were subjected to HPLC in order to determine the impurities in the isolated compound.

Instrument **:** Lachrom L –700 Series

Solvent system: Dissolve 15.6g of dibasic sodium phosphate and 12.2 gms of monobasic potassium

Flow rate **:** 0.6ml/min.

Retention time **:** 13.45

Percentage purity: 95.56%

- **IR SPECTRAL DATA:**

Table: 21 **IR Spectral Details of isolated sterol S_8**

S.No	FREQUENCY cm-1	STRETCHING & DEFORMATION
01	3318	OH STRETCHING
02	2958-2850	-C-H STRETCHING
03	2728-1727	-C=O STRETCHING IN STERIOD ESTER
04	1666	-C-C MULTIPLE BOND STRETCHING
05	1600-1455	-C-CH3 STRETCHING
06	1378	-C-H DEFORMATION
07	1281	-C-O STRETCHING
08	1218-1128	PHENOLIC OH STRECTHING
09	1037	-C-C STRETCHING
10	836-666	Aromatic Substitution

- *NMR SPECTRAL DATA: PMR*

Table: 22 **NMR (PMR) Spectral Details of isolated sterol S_8**

S.No	SIGNALS (δ) Values ppm	GROUP ASSIGNED
01	7.7-7.5	Aryl Proton
02	5.4	Aromatic OH Proton
03	4.2	Alkyl OH Proton
04	2.0	CH-C=O Proton
05	1.7-1.6	Non conjugated Proton
06	0.7-1.5	Methyl Proton

- *NMR SPECTRAL DATA: ^{13}C NMR*

Table: 23 **NMR (^{13}C NMR) Spectral Details of isolated sterol S_8**

S.No	SIGNALS (δ) Values ppm	GROUP ASSIGNED
01	162	Carbonyl group attached to aromatic ring followed by CH_2
02	130-125	Aromatic
03	129	Double bond in aromatic
04	072	Solvent peak due to $CDCl_3$
05	032	C-8
06	31.6	C-2
07	028	C-25
08	24.3	C-15
09	24.1	C-22
10	022	C-26 Carbon attached to any group
11	018	C-21 (Methyl group carbon)
12	12 & 19.4	Indicates C_{18} & C_{19} in sterol ring

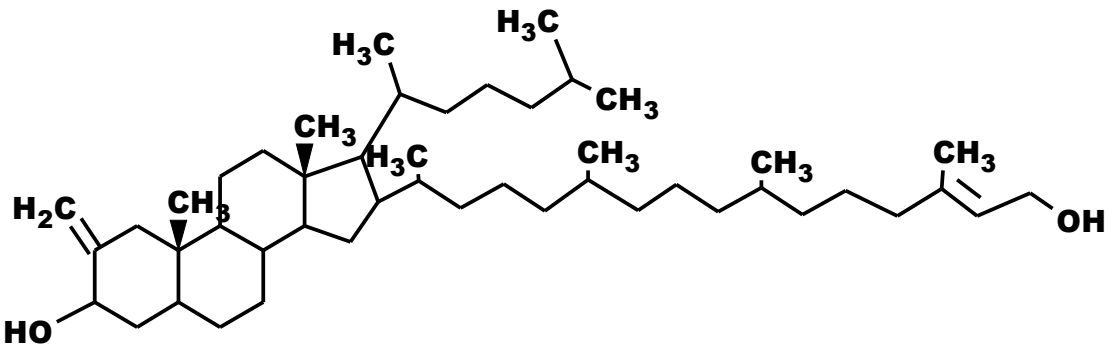
- **MASS SPECTRAL DATA**

Table : 24 **Mass Spectral Details of isolated sterol S_8**

S.No	Compound	Formula	Molecular weight	Retention time
01	2-Pentadecanone,6,10,14-trimethyl-	$C_{18}H_{36}O$	268	3.05
02	Cholestan-3-ol, 2-methylene-, (3α, 5α)	$C_{28}H_{48}O$	400	3.50
03	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296	4.94
04	Mono(2-ethylexyl) phthalate	$C_{16}H_{22}O_4$	278	9.95
05	α- Amyrin	$C_{30}H_{50}O$	426	24.11
06	Lupeol	$C_{30}H_{50}O$	426	25.35

Based upon UV, IR, NMR and Mass spectral datas the isolated compound S_8 may be

16-(3,7,11,15- Tetramethyl-2-hexadecen-1-ol)-2-methylene (3α,5α)-Cholestan-3-ol.



5. 9. Isolated compound F_1

- COMPOUND CODE:**F_1**

- PHYSICAL EXAMINATION:

  - **Colour:** Flourescent Yellow
  - **State :** Amorphous powder

- IDENTIFCATION

a) Chemical tests: Shinoda test, Zinc Hydrochloride and Alkaline reagent test.

b) THIN LAYER CHROMATOGRAPHY:

- **Stationary phase :** Silica -gel GF 254
- **Mobile phase :**Benzene(9) : Acetone (1)
- **Detector :** UV 254/365
- **Colour :** Flourescent green.
- **R_f value :** 0.79

✸ PHYSICAL CONSTANT

Melting Point: 124℃

✸ SOLUBILITY:

Ethyl acetate and Methanol.

✸ YIELD: 100 **mg**

✸ UV SPECTRAL DATA:

λmax 365nm

✸ **IR SPECTRAL DATA:**

Table: 25 **IR Spectral Details of isolated Flavanoid F_1**

S.No	FREQUENCY cm-1	STRETCHING & DEFORMATION
01	3406-3322	OH STRETCHING
02	1665-1611	-C=O STRETCHING in lactone ring
03	1562	-CH-CH- STRETCHING
04	1522	-C=C STRETCHING
05	1451	-CH Deformation
06	1408	-C-O- STRETCHING
07	1382	-OH Bending &–C-O STRETCHING
08	1262-1197	Phenolic OH STRETCHING
09	722-680	Aromatic Substitution

✸ **NMR SPECTRAL DATA: PMR**

Table: 26 **NMR (PMR) Spectral Details of isolated Flavanoid F_1**

S.No	SIGNALS (δ) Values ppm	GROUP ASSIGNED
01	7.7	Aryl Proton
02	1.5	Methyl Proton

13. AIM AND OBJECTIVES OF THE PROPOSED WORK

***Delonix regia* (Boj. ex Hook.) Raf.** (Caesalpinaceae) is a well known ornamental tree. The tree is noted to be as one of the five among the world most beautiful ornamental tree. However the tree was not familiar for its medicinal properties. The *Delonix regia* (Boj. ex Hook.) Raf. said to possess very less pharmacological actions and still kept aside just as an ornamental plant only. Various research scholars and scientists tried to explore the plant for its phytoconstituents and pharmacological properties. A thorough literature survey through various journals, textbooks and internet sources had been made to study about the current status of the drug.

It is noted that, not much work had been done on the leaves of the tree. So, here a clear-cut attempt had been made to explore the phytoconstituents of the same through various phytochemical methods. The isolated compounds were characterized using various spectroscopical studies such as UV, IR, NMR and Mass spectroscopical methods.

Free radical have aroused significant interest among scientist in past decade, their broad range of effect in biological systems have drawn on the attention of many experimental works. There are extensive evidences to implicate free radicals in the development of degenerative diseases such as cancer, cardiovascular diseases, rheumatoid arthritis, cataracts, diabetes, alzheimers disease and aging. It is suggested that free radical damage to cells leads to the pathological changes associated with many diseases and the antioxidant mechanism may be important in controlling the pathogenesis of certain diseases. Many synthetic antioxidant components have shown to produce toxic or mutagenic effects, which have change the attention towards the naturally occurring antioxidants.

The isolated compounds from ***Delonix regia*** (Boj. ex Hook.) Raf. leaves were subjected to the antioxidant screening models at various concentrations both by in vitro and ex vivo methods and tried to found out that whether they possess any such properties and if so, which one is better than the other.

The whole world atmosphere is polluted by means of microbes. One or the other way they also form as an integral part of the human society itself. They are said to causes so many disastrous diseases and infections such as Tuberculosis, HIV, fever and the list goes on and on. So in order to check, whether our drugs as such possess any antimicrobial effect, the alcoholic extract of ***Delonix regia*** (Boj. ex Hook.) Raf was screened against microbes such as gram positive bacteria, gram negative bacteria and fungus.

14. THE RESULTS & DISCUSSION

In the present study from the leaves of *Delonix regia* (Boj. ex Hook.) Raf was subjected to phytochemical screening along with isolation of phytoconstituents. The isolated phytoconstituents were characterized by means of spectral studies and subjected to determine their antioxidant potential by means of in vitro and ex vivo methods. The crude extract of the plants were tried to determine their anti-microbial potential.

I. Phytochemical investigation

The qualitative chemical investigation was carried out to determine the phytoconstituents present in the leaves of the plant. The tests revealed that the drug may possess phytoconstituents such as alkaloids, flavanoids, glycosides, sterols, triterpenoids, tannins, carbohydrates and proteins. Further chromatographical studies were carried out to confirm the same.

II. Isolation of Phytoconstituents

Crude isolation of sterols were isolated and confirmed the same through phytochemical and chromatographical studies. A systematic approach had been made to isolate phytoconstituents such as sterols and flavanoids by means of column chromatography. Eight sterols and a flavanoid had been isolated and confirmed the same. The percentage yield of sterols were 1.8%,

4.2%, 0.09%, 0.10%, 1.75%, 0.045%, 1.8% and 3.15% respectively. Were as the percentage yield of flavanoid was found to be 0.67%.

III. Characterization of isolated phytoconstituents

The isolated phytoconstituents were further subjected various spectral studies such as UV, IR, NMR and Mass spectroscopy.

The isolated phytoconstituents when subjected to IR spectral studies shows the presence of - OH, -CH, -C=C-, -C=O, γ-lactone stretching and bending, aromatic substitution within them by exhibiting bands in their respective regions.

Further the isolated compounds were observed for their ^{1}H NMR & ^{13}CNMR which revealed the presence aryl, methyl, hydroxyl, carbonyl and secondary alkyl proton, which confirms the steroidal ring and lactonic ring.

To completely elucidate the structure of isolated sterol S_8 they were subjected to fragmentation by mass spectroscopical studies. At about the retention time 3.50 the sample exhibited a fragmentation with molecular weight 400 and molecular formula $C_{28}H_{48}O$, so the compound S_8 may be a cholestanol derivative. In the same spectra at about retention time 4.94 there was a long chain linear fragment with molecular weight 296 and formula $C_{20}H_{40}O$.

Based upon this the compound may be named as **16-(3,7,11,15-Tetramethyl-2-hexadecen-1-ol)-2-methylene (3α, 5α)- Cholestan-3-ol .**

IV. <u>Physico & Chemcial evaluation</u>

Delonix regia (Boj. ex Hook.) Raf. leaves were subjected to ash values, extractive values and elemental analysis. Total ash of 07.4% and sulphated ash of 07.19% ash were observed. The various extractive values carried out shown the maximum percentage yield of 47.80% of water soluble extractives and minimum percentage yield of 02.938% in n-Hexane soluble extractives. The elemental content such as sodium, potassium and Calcium were determined and by means of flame photometer and were found to be 0.5 mEq, 0.1 mEq and 1.0mEq respectively.

V. <u>Pharmacological evaluation</u>

a. Antimicrobia potential of *Delonix regia* (Boj. ex Hook.) Raf

The antibacterial and antifungal activity of *Delonix regia* (Boj. ex Hook.) Raf was evaluated by means of using the following Gram positive, Gram negative and fungus: *Strepto cocci, Staphylo cocci, Proteus vulgaris, Escherichia coli, Pseudomonas aeuroginosa, Klebsiella aerugenes, Candida albicans.* The activities were evaluated at about various concentrations of 500µg, 750µg, 1000µg & 2000µg. Amikacin 5µg and Ketoconazole 10µg

were used as the standards for bacteria and fungus. The extracts had shown moderate antibacterial activity when compared with the standard at about a dose of 2000µg. The maximum antibacterial activity was observed against Pseudomonas aeuroginosa, which was highly competitive with the standard. The extract had shown good anti-fungal activity at the highest concentration of 2000µg.

b. Estimation of Total Phenolics: The percentage of total Phenolics were determined to be 19.8845 mg/100gm as gallic acid equivalent which serves as the preliminary estimation of antioxidant potential.

c. Antioxidant potential of isolated phytoconstituents

The isolated sterol and Flavanoid were subjected to antioxidant evaluation using reducing power ability, DPPH radical scavenging assay, Nitric oxide radical scavenging assay and Lipid peroxidation assay. The isolated compounds at about 5 different concentrations of 125µg/ml, 250µg/ml, 500µg/ml, 750µg/ml and 1000µg/ml were screened for their antioxidant properties using ascorbic acid as standard at 01µg/ml, 02µg/ml, 03µg/ml, 04µg/m1, 5µg/ml for all the models except lipid peroxidation assay. For lipid peroxidation assay ascorbic acid was used at 25µg/ml, 50µg/ml, 100µg/ml, 250µg/ml & 500µg/ml concentrations.

The isolated compounds were shown good reductive ability with increasing concentrations which were comparable to the standard. The maximum reduction in absorbance was absorbed at 1000µg/ml for both the isolated compound and for the standard at 5µg/ml.

DPPH radical scavenging assay, Nitric oxide radical scavenging assay and Lipid peroxidation assay were carried out and their IC_{50} values were determined for the samples and standards. Based on the IC_{50} values of the isolated compound it is found that they possess significant anti-oxidant potential as compared to the standard.

SUMMARY & CONCLUSION

The present study of D*elonix regia* (Boj. Ex Hook.) Raf aims at exploring the phytoconstituents of it and to characterize them along with their antioxidant potential. Further it also tries to justify the antimicrobial properties of the leaves of the tree. Isolation of sterols and flavanoid was done and maximum possibility of characterization of isolated sterol was carried out. The isolated compound sterol may be found to be as **16 - (3, 7, 11, 15 – Tetramethyl – 2 – hexadecen – 1 - ol) – 2 - methylene (3α, 5α)-Cholestan – 3 - ol.** The anti-microbial study of the leave extract was carried out and compared with the standard. The drug is found to be potential against *Pseudomonas aeruginosa* and the fungus *Candida albicans*. The isolated sterol and flavanoid were evaluated for their antioxidant properties using ascorbic acid as standard. Both the isolated compounds found to possess significant antioxidant potential and as compared to the standard. However, the antioxidant potential of flavanoid was found to be more than that of sterol. These were concluded by means of the lower IC_{50} values of flavanoid than sterol. It can be concluded that the D*elonix regia* (Boj. Ex Hook.) Raf, which is known better for its ornamental properties, can be explored for its pharmacologically active marker phytoconstituents in future.

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